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The third year of the University of Michigan Human Breast Cell/Tissue Bank and Data base has been dedicated to providing investigators from around the country and internationally with the cells and tissues we have banked and with new breast cancer cell lines developed in our lab. A World Wide Web page, which contains an on-line order form that allows investigators to request the cells or tissues that suits their needs, has been visited over 1000 times. Many investigators have made use of the on-line order form for requests for breast cancer cells and tissues. Thus, over the past year, our core has distributed frozen human breast cancer cells, breast cancer cell lines, and paraffin embedded and frozen histologic sections from breast cancer specimens to 33 different laboratories. Also during this year, a complete genetic analysis of the new breast cancer cell lines was carried out and that data are available to investigators using the cell lines. Goals for the upcoming year are to improve the visibility of this resource in order to increase the number of requests that we receive and fill.

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Stephen P. Ethier 9-26-97

PI - Signature Date

Stephen Ethier, Ph.D.

Human Breast Cancer Cell/Tissue Bank and Database

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INTRODUCTION

The major goal of the work that is supported by this DOD infrastructure grant is to develop a human breast cancer cell/tissue bank and data base to facilitate distribution of fresh breast cancer specimens to breast cancer researchers in our own institution and throughout the country. Many aspects of our resource are unique. For example, we are the only such tissue bank that provides both normal and neoplastic breast epithelial cells in a viable state suitable for in vitro studies as well as for the more common molecular biological applications. We are providing frozen sections and touch preps in addition to the more common paraffin embedded sections. We are providing clinical data on each patient sample that includes the common clinical information, such as estrogen and progesterone receptor statues and lymph node status. We also provide data on expression of certain oncogenes and tumor suppresser genes (erbB-2 and P53 expression status) in these samples. Finally, we are actively establishing new human breast cancer cell lines which we provide to the scientific community. Our cell lines are cultured under growth factor-defined conditions and come with a full array of cellular and molecular data. Furthermore, cells from these lines are provided at early passage levels, thus, providing a better model of breast cancer cell growth in vivo. To data, we have established 13 new human breast cancer cell lines from early and late stage primary tumors and from metastatic sites.

Thus, we have developed a unique human breast cell/tissue bank and data base that is providing important breast cancer cells and tissues to many breast cancer researchers.

BODY

During the past year, this core facility has been fully operational and has extended the work that was done in the previous year. All aspects of our core facility are functional and we have provided a variety of specimen types to breast cancer researchers all over the world. Individual aspects will be discussed in terms of the tasks as outlined in the original SOW.

Task 1. Establish logistic methods for insuring that every human breast cancer specimen that has tissue remaining following evaluation by the Surgical Pathologist is routed to the Cancer Biology laboratory for tissue preparation, cell isolation and cyropreservation in the cell bank.

Most of the work relevant to this task was accomplished during year one of the grant. Thus, by the beginning of year 2, the mechanisms were already in place to insure that all new human breast cancer specimens became part of our growing cell and tissue resource. A tissue procurement technician routinely obtains all biopsied breast cancer material from the surgical pathology suite of the operating room, and with the approval of a staff pathologist, obtains any non-used tissue and directs it to the cell biology laboratory for breast cancer cell isolation, banking and cell line development. For tumors of sufficient size, solid pieces are frozen directly in liquid nitrogen for the preparation of touch preps and frozen sections. Finally, all paraffin

embedded breast cancers are included in the bank of tumors available for the preparation of new histologic sections. All of this continued without change or interruption in year three.

At the present time, there are breast cancer specimens from 309 patients banked as paraffin embedded tissues, for which clinical, histopathologic and immunohistochemical data are available. This number represents an increase of 40 patients from last year. The frozen bank of isolated human breast cancer cells now consists of 800 ampoules of cells from 164 different patients. Specimens from 38 patients were added to the bank during year three Also, during the past year, three new human breast cancer cell lines have been established, bringing the total developed in this laboratory to thirteen. All of these lines are part of this resource and many of these lines have been distributed to investigators.

In addition to developing new human breast cancer cell lines, all of our lines were extensively characterized genetically over the past year. In collaboration with Dr. Olli Kallioniemi, we performed comparative genomic hybridization analysis on all of our cell lines, and complimented that analysis with Southern blot experiments to confirm the amplifications of specific genes. Thus, we now have mapped the regions of gene amplification and gene loss in all of these cell lines. In addition to the genetic studies, further work to characterize these lines at the cellular level has been carried out. Thus, we have studied the role of constitutive activation of pp125-focal adhesion kinase, and STAT3 in these breast cancer cell lines. Results of these experiments have been published and others have been submitted for publication. Data from the genetic analysis and reprints/preprints of current papers are included in the appendix.

Thus, the knowledge base surrounding this unique panel of human breast cancer cell lines in growing rapidly and will, no doubt, contribute to a better understanding of the cellular and molecular alterations important in human breast cancer development.

Task 2. Establish methods for histologic evaluation of parameters not ordinarily evaluated for human breast cancer specimens, for all specimens that yield cells that are stored in the tissue bank. These include immunohistochemical evaluation of expression levels of p53 protein, EGFR receptor and HER-2/neu receptor. Progress toward this task was discussed briefly above. In addition to routine histopathologic analysis done on all breast cancer specimens, all breast cancer specimens that are part of our cell/tissue bank are evaluated separately for expression of erbB-2 and p53. As mentioned above, we now have 309 banked breast cancer specimens with data on erbB-2 and p53, in addition to the routine histopathologic information.

Task 3. Establish logistic methods for routine blood drawing of all breast cancer patients whose cells are preserved in the bank, in order to isolate and immortalize lymphocytes from these patients. We now have in place all of the logistic and technical facilities required to

provide immortalized lymphocytes for investigators that require these cells for matched normal DNA. A large bank of immortalized lymphocytes already exists here as part of the breast cancer family history data base and these cells can be used and distributed to investigators interested in this patient set. Additionally, we have the ability to draw blood from patients, and isolate and immortalize the lyphocytes on an as-needed basis. To date, few investigators have demonstrated an interest in this aspect of our core. Thus, we continue to do this only in special cases as requested by investigators.

Task 4. Establish a computerized data base for all patients whose cells are currently stored in the cell bank and for all future patients whose cells/tissues are banked. The data base will contain all pertinent family history data, all data obtained from histopathologic evaluation of the breast cancer specimen, the location and status of the patients' cells and tissues stored in the bank. Experimental data obtained by individual investigators using banked samples will also be entered into the data base. The data base will be set-up in a way that allows investigators to access patient data without compromising the privacy and confidentiality of the patient.

The current data base is divided into sections that contain; demographic data for all patients whose cells and tissues are banked, pathological data for all specimens, data on the availability of fresh cells, and family history data for patients whose cells and tissues have been banked. The various parts of the data base are connected in ways that allow multiparameter searches of our entire data base. This allows us to provide the kinds of specimens that investigators request for specific subsets of patient samples. We have a fully operational data base that contains all of the data in one central location.

During the construction of this data base, many precautions were taken to protect the identity of patients whose cells/tissues are banked. First, the server that contains the data base is heavily password protected. In addition, each patient sample in the bank is assigned a unique number, referred to as a Key ID, that cannot be linked to the patients identity. Thus, any clinical data that is provided with the specimen is only linked to that unique number.

The data base, as it now stands, is a powerful supplement to the cell/tissue resource and allows us to fulfill specific needs of many breast cancer researchers. A data manager is supported by this grant, who's major task is to integrate all of the data on the specimens in our bank and keep the central data base up-to-date. In addition, all requests for cells and tissues are routed to this data manager who keeps the data base current, and is also responsible for obtaining appropriate data that is given to investigators along with the cells or tissues they receive. Thus, the data base is fully prepared to find specimens within our growing bank of cells and tissues that suit the needs of specific investigators.

Task 5. Set up and implement the administrative plan for distribution of cells and tissues stored in the bank to other investigators at the University of Michigan as well as other Cancer Centers throughout the mid-west and the country. We developed an ordering procedure for the distribution of cells and tissues in the bank. The order form that was developed sets limits on availability of various specimen types. In addition, we set up priority criteria that drive decisions to disburse cells/tissues in the event that availability of particular specimens becomes limited.

During year three, we continued to advertise the availability of cells and tissues from this resource. We did by preparing handouts that were distributed at breast cancer scientific meetings attended by the P.I. during 1996. Also during the past year, we placed advertisements in leading cancer journals. Our world wide web site continues to be our main contact with the scientific community. This web site contains a menu of cells and tissues available from the resource as well as an on-line request form. Investigators can fill out the form on line, and by clicking on the hypertext "send" can route the order to the P.I. via e-mail. Recently, the web site was linked to other breast cancer sites and data bases, including the web site set up by the National Breast Cancer action plan. The URL for our web site is:

http://www.cancer.med.umich.edu/umbnkdb.html

These approaches have resulted in our receiving several requests for cells and tissues during this year. The web site has been visited over 1000 times. We have received 63 requests for cells and tissues during year 3.

20 of these requests were directed to the Pathology group. To fill these requests the Pathology group prepared 334 haematoxylin and eosin stained sections from paraffin embedded tissues and 1,136 non-stained sections. In addition, 134 frozen sections were prepared and distributed.

A total of 43 requests for cells and cell lines came to the Cell Biology group during the past year. To fill these requests, 93 ampoules of frozen breast cancer cells, 129 ampoules of breast cancer cell lines, and 27 ampoules of normal human mammary epithelial cells were distributed to various investigators.

A series of tables are presented in the appendix which give details on the specimens disbursed from our resource during year 3 of the grant, as well as a list of the investigators who have received breast cancer cells and tissues from us.

CONCLUSIONS

In summary, the third year of this project has been quite successful and all of the tasks outlined in the Statement of Work have been completed and our core facility is fully operational. We feel strongly that the cell and tissue bank that we have developed provides many unique features to breast cancer researchers and will make a strong contribution to many areas of breast

cancer research. The main goal for the next year of this project is to increase the visibility of this resource so as to increase significantly the number of requests for cells and tissues that we receive and fill. This will be done by placing ads in selected scientific journals and by direct mailings to individuals whose focus is breast cancer research. The infrastructure is clearly in place and we have demonstrated that we can provide breast cancer cells and tissues to investigators.

As we enter into the final year of this grant, we must now begin to consider what will become of this resource once the DOD support lapses. Certainly, the U of M Cancer Center can continue to support some aspects of this core facility and it will be possible to begin to charge investigators for specimens that they receive for us. However, the development of new human breast cancer cell lines, a unique and important feature of our resource, will be difficult to maintain without specific funding for this purpose. We strongly urge the U.S. Army Research and Materiel Command to consider a mechanism for the continued funding of successful and important infrastructures that resulted from the original DOD breast cancer program.

REFERENCES

none.

Appendix

1. Summary of disbursement of tissues by Pathology

2. Summary of disbursement of cells and cell lines by Cancer Biology

3. List of investigators receiving cells or tissues

4. Summary of genetic analysis of human breast cancer cell lines developed in our laboratory

    - Summary of comparative genomic hybridization (CGH) analysis
    - Summary of oncogene amplification in "SUM" lines
    - Summary of high level amplifications in SUM lines
    - Overall CGH results for SUM lines

5. Relevant publications

1996 - 1997 Database Report

Total Number of Patients in the Database = 309
1996 - 1997 Additions = 40

There were **63 requests** from Principal Investigators to the Database
61 from the United States
(CA:10; CT:2; IL: 2; IN: 1; LA: 3; MA: 11; MD: 6; MI: 9; NJ: 1; NY: 4; OH: 2; OR: 1; PA: 6; TX: 3)
2 from outside the United States
(Italy: 2)

Pathology

There were **20 requests** filled by Pathology
There were 1,604 specimens sent to those twenty Investigators:

Requests fulfilled by Pathology 1995-96

Paraffin:
334 Haematoxylin & Eosin (H/E)
1,136 silane slides (plus slides)

Frozen:
134 Frozen sections

There were no Touch preps requested or given.
All of the specimens were accompanied by Pathological Data.

	Requests fulfilled by Pathology 1996-1997					
Date	Ca (H&E)	nl (H&E)	Ca (+)	nl (+)	F Sec	Total(by month)
Oct-96	6	6	18	18	18	66
Nov-96	73	74	150	171	44	512
Dec-96						
Jan-97	15		30	30	45	120
Feb-97	20		60			80
Mar-97	20		185			205
Apr-97	22	22	34	44		122
May-97	12	5	74	18		109
Jun-97	8		64			72
Jul-97	8		64			72
Aug-97	41	2	176		27	246
Sep-97						
Total	225	109	855	281	134	1604

Abbreviations used in Table:

Ca (H & E)	- Cancer specimen on a Haematoxylin & Eosin slide
Nl (H & E)	- Normal specimen on a Haematoxylin & Eosin slide
Ca (+)	- Cancer specimen on a silane slide (plus slide)
Nl (+)	- Normal specimen on a silane slide (plus slide)
F Sec	- Frozen section of a breast cancer tumor

Cancer Biology

There were **43 requests** filled by Cancer Biology.
From the 43 requests made, **249 Samples** were distributed (Frozen segments, HME's, cell lines).

Number of Frozen ampules and HME's 1996-97

Frozen ampules :		Human Mammary Epithelial:	
October 1996:		October 1996:	
November:	6	November:	2
December:	12	December:	
January 1997:		January 1997:	
February:	66	February:	12
March:		March:	
April:		April:	2
May:		May:	2
June:	2	June:	2
July:		July:	
August:	7	August:	7
September:		September:	
Total:	**93**		**27**

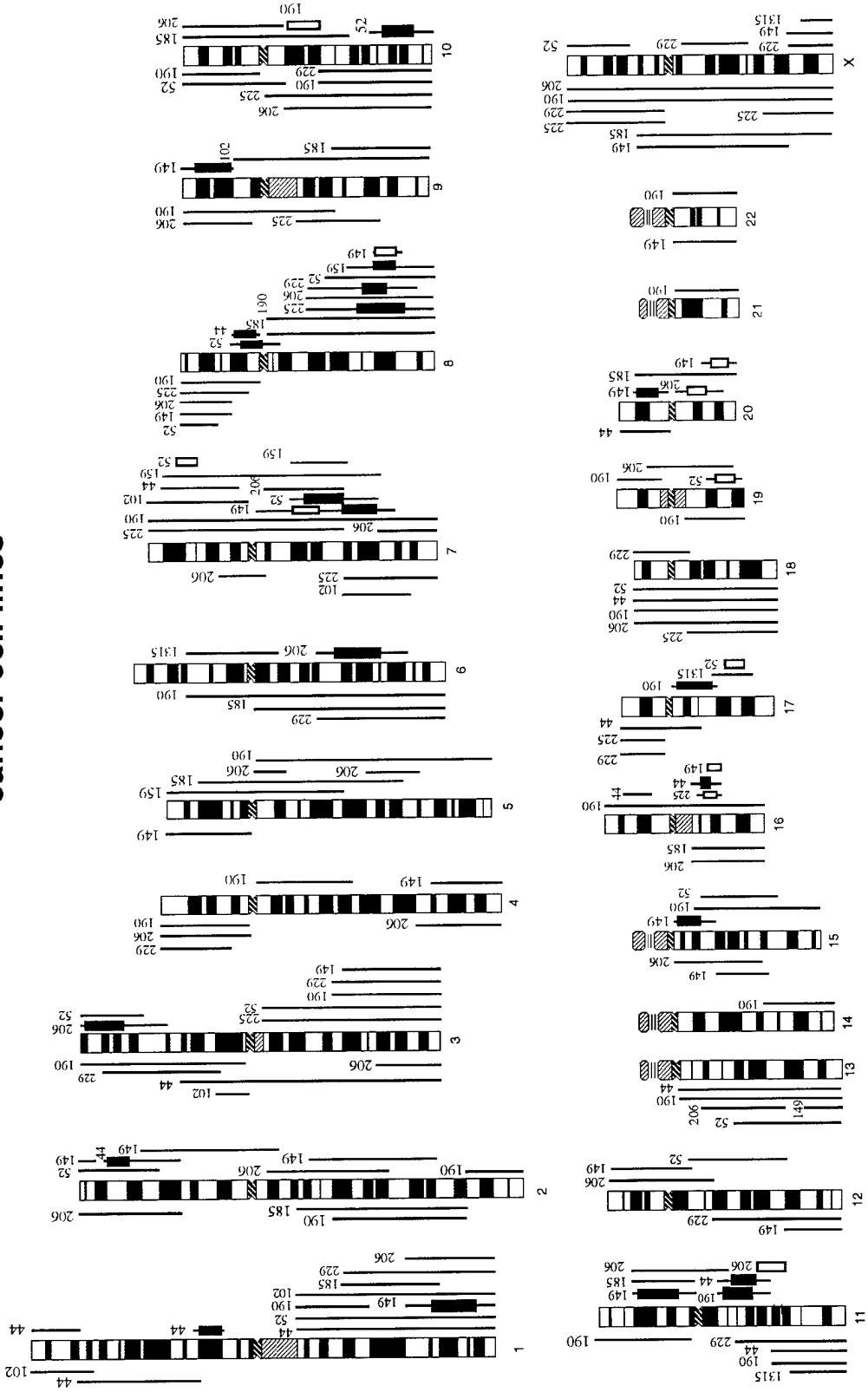
From October 1996 to the present, there has been an increase of 3 new cell lines
Below is the breakdown of the distribution of cell lines.

Distribution of Cell lines												
Date	**SUM-44PE**	**SUM-52PE**	**SUM102PT**	**SUM-149PT**	**SUM-1315MO2**	**SUM-159PT**	**SUM 185**	**SUM 190**	**SUM 206**	**SUM 225**	**SUM 229**	**Total**
Oct-96	3	3	3	2	2	1	1	1	1			17
Nov-96	1	1	1	1	1	1						6
Dec-96	1	4	1	1	1	1						9
Jan-97												
Feb-97	2	3	2	3		1						11
Mar-97												
Apr-97	6	3	7	1		2						19
May-97	1	1	1			1						4
Jun-97	4	4	1	2		2		2	1		1	17
Jul-97	1	2		2	1	2	1	3				12
Aug-97	4	3		4	1	3	3	5	5	2	3	33
Sep-97					1							1
Total	23	24	16	16	7	14	5	11	7	2	4	129

Principal Investigators 1996-97

Last name	First name	Institution	City	State	Country
Atkins	Kevin	University of Michigan	Ann Arbor	MI	USA
Clevenger	Charles	University of Pennsylvania	Philadelphia	PA	USA
Dorfman	David	Harvard Brigham and Women's Hospital	Boston	MA	USA
Gudas	Jean	Amgen, Inc.	Thousand Oaks	CA	USA
Hoover	Kevin	University of California	Irvine	CA	USA
Jaken	Susan	Waj Cell Science Center	Lake Placid	NY	USA
Kahan	Zsuzsanna	Tulane University Medical Center	New Orleans	LA	USA
Kallioniemi	Olli	National Center for Human Genome Research	Bethesda	MD	USA
Kapoun	Ann	Children's Hospital Los Angeles	Los Angeles	CA	USA
Karnik	Pratima	Cleveland Clinic	Cleveland	OH	USA
Kefalides	Nicholas	University of Pennsylvania	Philadelphia	PA	USA
Kurnit	David	University of Michigan	Ann Arbor	MI	USA
Kurt	Robert	Earle Chilles Research Institute	Portland	OR	USA
Lowe	Scott	Cold Spring Harbor Laboratory	Cold Spring Harbor	NY	USA
Merajver	Sofia	University of Michigan	Ann Arbor	MI	USA
Moses	Marsha	Children's Hospital-Harvard University	Boston	MA	USA
Nakshatri	Harikrishna	Indiana University	Indianapolis	IN	USA
Niedbala	Michael	Bayer Research Center	West Haven	CT	USA
Ostrand-Rosenberg	Suzanne	University of Maryland	Baltimore	MD	USA
Rotenberg	Susan	Queens College	Flushing	NY	USA
Sarraf	Pasha	Dana Farber Cancer Institute	Boston	MA	USA
Shackleford	Gregory	Children's Hospital Los Angeles	Los Angeles	CA	USA
Strayer	David	Thomas Jefferson University	Philadelphia	PA	USA
Svoboda-Newman	Suzette	University of Michigan	Ann Arbor	MI	USA
Weigel	Ron	Stanford University	Stanford	CA	USA
Welch	Danny	Pennsylvania State Medical College	Hershey	PA	USA
Wong	Tai Wai	Bristol Myers Quibb Pharm Res Institute	Princeton	NJ	USA
Woodruff	Teresa	Northwestern University	Chicago	IL	USA
Wu	Inmin	Children's Hospital-Harvard University	Boston	MA	USA
Xian-Feng	Zhang	Harvard University	Charlestown	MA	USA
Yazdi	Youseph	University of Texas at Austin	Austin	TX	USA
Yu	Ben	Henry Ford Health System	Detroit	MI	USA
Zucchi	Ileana	Istituto Technologie Biomediche Avanzate	Milano		ITALY

Ideogram summary of gains (right) and losses (left) in eleven SUM breast cancer cell lines



*Black boxes represent regions of high level amplification by CGH and the clear boxes putative areas of low level amplification.

	FISH RESULTS					
	Cyclin D1	**11 centromere**	**c-MYC**	**8 centromere**	**ERBB2**	**17 centromere**
Sum 206	4	5	6	6	4	3
Sum 1315	4	5	2	5	3	3
Sum 190	**>14**	**2**	7	8	**>32**	**8**
Sum 159	2	2	5	2	3	2
Sum 44	**>17**	**8**	**7**	**4**	**>39**	6
Sum 149	4	4	5	4	3	2
Sum 52	**14**	**5**	11	12	4	4
Sum 185	6	5	8	7	4	3
Sum 102	4	3	6	4	4	4
Sum 225	11	6	**12**	5	**>56**	7
Sum 229	9	6	**7**	4	7	5

High level amplifications (by CGH) (>=1.4) (recurrent in bold)					
Sum 206	**1q41-44**, 3p26-22, 6q21-24, 7q36, **8q22-24.3**				
Sum 190	3q26.2-29, **8q21.1-22**, **11q13-14**, 14q32, 15q25,				
	16p13.1-13.2,16q22-24, 17q11.2-21, 21q21-22,				
	22q11.1-12				
Sum 159	5p15, **8q23**				
Sum 44	**8p11.2-12**, **11q13-14**				
Sum 149	**1q32-43**, 4q32-35, 7q31, 9p21-23, 12p12, 12p11.2-12				
Sum 52	7q21-22, **8p11.2**, **8q23-24.1**, 10q24-26, 12q21				
Sum 185	9q31-34, 10p13-15, 20q11.2				
Sum 229	**8q23-q24**				

Overall CGH RESULTS of eleven cell lines			
Most common Gains		Most common Losses	
Region	Frequency (%)	Region	Frequency (%)
1q(32)	64	Xq(cent.-11.3)	54
7q(21-22)	64	8p(22-23)	45
8q(22-24.1)	64	18q(12-22)	45
3q(24-29)	45	Xp(24-25)	45
7p(12-21)	45	10q(22-26)	36
5q(11.2-12)	36	11q(23-25)	36
2p(21)	27	13q(21-22)	36
11p(cent.-14)	27	13q(24-26)	36
11q(13)	27	18p	36
15q(15)	27	4p (14-16)	27
20q(13)	27	3p(12)	27
Xq(27-28)	27	3p(13-14)	27
		6q(21-27)	27
		17p	27

[CANCER RESEARCH 57, 978–987, March 1, 1997]

Role of Epidermal Growth Factor Receptor and STAT-3 Activation in Autonomous Proliferation of SUM-102PT Human Breast Cancer Cells[1]

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ABSTRACT

This report describes the isolation and characterization of a new human breast cancer cell line, SUM-102PT, obtained from a minimally invasive human breast carcinoma. SUM-102PT cells have a near diploid karyotype, and early-passage cells had minor chromosomal abnormalities including a 5, 12 and a 6, 16 reciprocal translocation. The cells were isolated and have been continually cultured in three defined media, one of which contains exogenous epidermal growth factor (EGF). SUM-102PT cells have also been carried in an EGF-free medium supplemented with progesterone. All SUM-102PT cells require EGF receptor (EGFR) activation for continuous growth, because incubation of the cells with EGFR-neutralizing antibodies or with EGFR kinase inhibitors blocks growth of these cells. Southern analysis indicates that the *EGFR* gene is not amplified in these cells; however, these cells express high levels of EGFR mRNA. Thus, SUM-102PT is representative of a class of human breast cancers characterized by high level EGFR expression in the absence of gene amplification. SUM-102PT cells cultured in EGF-free, progesterone-containing medium express high levels of constitutively active EGFR. Conditioned medium from SUM-102PT cells contains an EGF-like mitogen that binds to a heparin-agarose affinity matrix with high affinity. Northern analysis for various EGF family members indicates that SUM-102PT cells synthesize heparin binding (HB)-EGF mRNA. HB-EGF protein is detectable on the surface of these cells by immunohistochemistry, and SUM-102PT cells are killed by diphtheria toxin, which acts by binding to HB-EGF. Furthermore, HB-EGF antibodies partially neutralize the mitogenic activity of the conditioned medium. Thus, EGFR activation in SUM-102PT cells is mediated, at least in part, by autocrine/juxtacrine stimulation by HB-EGF. SUM-102PT cells also express constitutively active STAT-3 homodimers. Constitutively tyrosine-phosphorylated STAT-3 homodimers were also detected in another breast cancer cell line, MDA468, which has an EGFR amplification and also has constitutive EGFR activity. Thus, SUM-102PT is a new human breast cancer cell line that expresses activated EGFR as a result of an autocrine/juxtacrine interaction with HB-EGF which, in turn, results in activation of STAT-3.

INTRODUCTION

Over the past several years our understanding of the molecular biology of human breast cancer has improved steadily. Several oncogenes and tumor suppressor genes have been shown to play important roles in breast cancer progression including *erb*B-2, c-*myc, p53,* and others (1–4). Although it is clear that molecular changes associated with the development of breast cancer result in alterations in growth-regulatory mechanisms of the affected cells, the precise cellular phenotypes that result from these genetic changes are understood poorly. Thus, it remains to be elucidated how specific molecular alterations by themselves, and in cooperation with other genetic changes, work to completely transform normal cells into cells with fully malignant properties.

One obstacle to a more clear understanding of the cellular consequences of molecular changes that occur in breast cancer has been the relative inability to culture primary breast cancer cells under well-defined conditions *in vitro*. This inability to isolate and culture primary human breast cancer cells is more reflective of a poor understanding of breast cancer cell biology than inadequacies of tissue culture technology, because one can routinely culture normal human mammary epithelial cells of both the luminal and basal/myoepithelial lineages (5–10). Furthermore, normal human mammary epithelial cells can be induced to express many differentiated functions *in vitro* by culturing them in appropriate matrices (11–13). Thus, normal human mammary epithelial cells can be induced to grow and differentiate in culture under well-defined conditions.

A major focus of our laboratory over the past several years has been to improve our understanding of the altered growth-regulatory pathways that distinguish human breast cancer cells from their normal counterparts. Accordingly, we have developed and continue to develop culture conditions for the growth of primary and metastatic human breast cancer cells (10, 14, 15). The purpose of these studies is not simply to isolate new human breast cancer cell lines but rather to isolate and culture human breast cancer cells under defined conditions to understand better how specific molecular alterations result in altered growth regulation of the cells. In this report, we describe a newly isolated breast cancer cell line, SUM-102PT, which was isolated from a minimally invasive primary human breast cancer. These cells have only minor karyotypic abnormalities, do not have amplifications of the known breast cancer oncogenes, but do overexpress EGFR[3] to high levels at both the RNA and protein levels. SUM-102PT cells also synthesize HB-EGF, which acts as an autocrine/juxtacrine mitogen for these cells. SUM-102PT cells also express a constitutively activated STAT-3 complex. This complex was not observed in MCF-10A normal mammary epithelial cells, which also depend on EGFR activation for growth.

Thus, the SUM-102PT cell line is a representative of a large subset of human breast cancers characterized by EGFR overexpression. Several clinical studies have demonstrated EGFR overexpression, in the absence of gene amplification, to be a bad prognostic indicator in human breast cancer (16–23). Thus, SUM-102PT may be a good model cell line for early stages of this important subset of human breast cancer.

MATERIALS AND METHODS

SUM-102PT Isolation. One-half g of the primary tumor specimen was obtained by our laboratory. The tumor was minced into fine pieces using two scalpels in a cross-cutting manner. The pieces were then placed in a 50-ml centrifuge tube containing 20 ml of Medium 199 and vortexed by hand, allowing the smaller cell clusters to remain in suspension and the larger pieces to settle to the bottom of the tube. The smaller cell clusters and single cells in

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; HB, heparin binding; DT, diphtheria toxin; SF, serum-free; SIE, serum-inducible element; STAT, signal transducer and activator of transcription.

suspension were centrifuged at 1000 rpm for 5 min and plated in 6-well culture plates in various culture media described below. The larger cell clusters were subjected to enzymatic dissociation by incubating the fragments in 15 ml of Medium 199 containing 0.1% Dispase (Boehringer Mannheim, Indianapolis, IN) and type III collagenase (Worthington Biochemical Corp., Freehold, NJ) at 200 units/ml overnight in a 65 cycles/min, 37°C shaking water bath. The cells were then washed three times with Medium 199, and nuclei were counted. Ten million cells from the enzymatic dissociation were then combined with 10^7 magnetic beads (Dynabeads; Dynal) conjugated to the monoclonal antibody MC-5 to further separate them from normal fibroblastic cells that are typically associated with a breast tumor specimen. The MC-5 antibody was kindly provided by Dr. J. Peterson (Cancer Research Fund of Contra Costa) and recognizes mucins expressed by human mammary epithelial cells. The cell-Dynabead mixture was allowed to agitate at room temperature for 2 h. The cells that bound to the MC-5-labeled Dynabeads were separated from the cell mixture using a magnetized tube holder. The purified mixture was then seeded onto 35-mm, 6-well culture dishes in media supplemented with different hormone and growth factor combinations as described below.

All media were prepared from a base of Ham's F-12 medium. Some media were supplemented with 5% fetal bovine serum. SF media were supplemented with 0.1% BSA, 50 ng/ml sodium selenite, 50 ng/ml 3,3′,5-triiodo-L-thyronine, 5 μg/ml transferrin, 5 mM ethanolamine, and buffered with 1 mM HEPES. Serum-containing and SF media were supplemented additionally with 1 μg/ml hydrocortisone, 10 ng/ml EGF, 5 μg/ml insulin, 0.1 μM progesterone, and 1 nM estradiol using the combinations shown in Table 1. Unless otherwise noted, all cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Assay for EGF-like Growth Factor Activity in Conditioned Medium. MCF10A cells were plated at a density of 2×10^4 cells/well in collagen-coated, six-well plates. They were then grown under normal conditions (SF medium containing EGF), in SF medium lacking EGF, or in SF/EGF-free medium with 48 h conditioned medium from SUM-102PT cells at 50% (v/v). After three feedings (1 week), nuclei were isolated and counted. For experiments aimed at modulating HB-EGF activity, SUM-102PT cells were plated at 2×10^4 cells per 35-mm well and cultured in SF-IHP medium supplemented additionally with either HB-EGF antibodies (R&D Systems, Minneapolis, MN) or with the non-toxic DT analogue, CRM-197. CRM-197 was a kind gift from Dr. Michael Klagsbrun. Cells were cultured for 7 days and counted as described above.

Heparin-Agarose Affinity Chromatography of SUM-102PT Conditioned Media. Forty-eight-h conditioned medium was collected from near-confluent cultures of SUM-102PT cells in SF-IH medium. Forty ml of conditioned media were passed over a 1.0-ml bed of heparin-agarose (Sigma Chemical Co., St. Louis, MO), and the flow-through media were collected. The column was then washed extensively with Tris-HCl (pH 7.4) and eluted with 2.5 ml of Tris-HCl containing either 0.2, 1.0, or 1.5 M NaCl. The eluates were desalted by diafiltration, filter sterilized, and tested for EGF-like activity as described above.

Northern Blot Analysis. Total RNA was prepared using guanidine isothiocyanate followed by organic extraction. Twenty μg of total RNA were electrophoresed through 1.5% agarose gels in formaldehyde, capillary blotted, and probed in formamide-containing hybridization buffer overnight at 42°C. The full-length EGFR probe was obtained from the American Type Culture Collection (Rockville, MD). The HB-EGF probe encompassed a 1.1-kb fragment beginning at bp 490 and was the kind gift of Dr. Stefan Stoll, as was the amphiregulin probe (900-bp 5′ fragment). Transforming growth factor α probe was a gift from Genentech. Final washes were 0.1× SSC, 0.1% SDS at 65°C. Gels were exposed to autoradiography for 24–96 h.

Immunocytochemical Analysis of HB-EGF and Cytokeratins. SUM-102PT cells near confluence were fixed with cold methanol. Fixed cells were incubated for 30 min with 1 μg of polyclonal rabbit anti-HB-EGF (R&D systems, Minneapolis, MN), followed by incubation with a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Inc. Burlingame, CA). Alternatively, fixed cells were immunostained with anti-cytokeratin antibodies: anti-pankeratin, anti-keratin-18, anti-keratin 8 (Sigma Immunochemicals, St. Louis, MO), and anti-keratin 19 (ICN Biomedical, Costa Mesa, CA). Cells were visualized using diaminobenzidine as a substrate for horseradish peroxidase according to the manufacturer's instructions (Vector Laboratories, Inc.).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously (24) by lysing cells under hypotonic conditions in buffer containing 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM sodium PP_i, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. Nuclei were isolated by centrifugation and lysed in the above buffer with 420 mM NaCl and 20% glycerol. Seven μg of nuclear proteins were incubated for 30 min at 30°C with end-labeled high-affinity SIE oligonucleotide (5′-AGCTTCATTTCCCGTAAATCCCTAAAGCT-3′) with or without 100-fold excess of unlabeled competitor or unlabeled scrambled competitor (5′-AGCTTAGCCTACCACTCTCTAA-3′). Complexes were separated through 7.5% nondenaturing PAGE. Gels were dried and autoradiographed 24–48 h. Supershifts were performed by incubating extracts for 20 min with 1 μg of anti-STAT-3 rabbit polyclonal antibody raised against peptides 750–769 of the COOH terminus (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Western Blot Analysis of Membrane Protein. Confluent monolayers of cells were scraped from 60-mm tissue culture dishes in 20 mM HEPES containing 5 mM sodium orthovanadate, 10 mM sodium PP_i, and 1 mM phenylmethylsulfonyl fluoride. The lysate was dounce homogenized 50 times and centrifuged at 800 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 35 min, and the pellet was resuspended in a buffer containing 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM sodium orthovanadate, 10 mM sodium PP_i, and 1 mM phenylmethylsulfonyl fluoride. The lysate was assayed for protein content, and then defined amounts of membrane protein were loaded into individual wells and electrophoresed on a 7.5% SDS-polyacrylamide gel. Following electrophoresis, the proteins were electroblotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA) and blocked with 3% nonfat dry milk in Tris-buffered saline with 1% Tween 20. The blot was probed with erbB-2 antibody (Pab 9.3, kindly provided by Dr. Beatrice Langton), anti-phosphotyrosine antibody (PY-20; ICN, Costa Mesa, CA), or EGFR antibody (Ciba-Corning, Alameda, CA). Protein bands were visualized by incubating blots with biotinylated secondary antibody and then with Vectastain ABC reagents (Vector Laboratories, Burlingame, CA) using diaminobenzidine as a substrate.

Table 1 *Media composition*[a]

Media Group	Growth factors/Supplements
Serum-containing media (5% fetal bovine serum)	
5% IH	Insulin, hydrocortisone
5% IHE	Insulin, hydrocortisone, EGF
SF media (0.1% BSA, ethanolamine, selenium, triiodothyronine, transferrin, and HEPES)	
SF-I	Insulin
SF-IP	Insulin, progesterone
SF-IH	Insulin, hydrocortisone
SF-IHP	Insulin, hydrocortisone, progesterone
SF-IHPE$_2$	Insulin, hydrocortisone, progesterone, estradiol

[a] All media also contain fungizone and gentimycin.

RESULTS

Isolation and Initial Characterization of SUM-102PT. SUM-102PT was isolated from a 56-year-old woman with locally advanced left breast cancer. Fine-needle aspiration cytology of the lesion was positive for adenocarcinoma, and the patient underwent 6 months of neoadjuvant chemotherapy with cytoxan, Adriamycin, methotrexate, and 5-fluorouracil prior to definitive surgery. Chemotherapy was followed by modified radical mastectomy, and the neoplasm was diagnosed as a minimally invasive apocrine adenocarcinoma with extensive ductal carcinoma *in situ*. Immunohistological analyses were negative for both estrogen and progesterone receptors. The tumor was histopathological grade 1 on the Bloom-Richardson scale, and none of the 14 lymph nodes contained tumor.

Cells were isolated from the primary tumor by enzymatic dissoci-

ation as described previously (10, 15). After dissociation, epithelial cells were separated from stromal cells by incubating the cell suspension with magnetic beads coated with MC-5 antibody, which recognizes breast epithelial mucins expressed by both normal and neoplastic breast epithelial cells (25). Purified epithelial cells were then seeded into 35-mm wells, and cultures were initiated in a variety of growth media.

We have recently developed several media that allow selective isolation of human breast cancer cells. However, cells from many breast cancer specimens do not grow, even in these media. In an attempt to develop other selective media, cells isolated from this specimen were cultured in the hormone- and growth factor-supplemented media listed in Table 1. From this specimen, proliferating cells were isolated using both 5% serum-containing and SF media that were supplemented additionally with insulin and hydrocortisone (IH medium), or with insulin, hydrocortisone, and progesterone (IHP medium). Interestingly, the cells cultured in the SF-IHP medium grew much more rapidly than the cells in the SF-IH medium, suggesting that progesterone had important effects on the growth of these cells.

Because of our interest in understanding growth regulation in human breast cancer cells, efforts were focused on cells cultured in the SF media. Since the early passages of SUM-102PT cells were cultured in EGF-free medium, experiments were carried out to determine if these cells were responsive to exogenous EGF. Cells cultured in SF-IH medium were cultured in the presence or absence of 10 ng/ml EGF. The data in Fig. 1 show that the addition of EGF to the culture medium increased the rate of proliferation of the SF-IH cells to a rate comparable to that of the cells cultured in SF-IHP medium. Thus, these cells, although not dependent on exogenous EGF for growth, are very responsive to this growth factor. From passage five onward, SUM-102PT cells were cultured continuously in three media: SF-IH, SF-IHP, and EGF-containing SF-IHE. SUM-102PT cells are immortal, have never exhibited signs of cellular senescence, and have been carried in culture for over 90 passages.

The isolation of immortal cell populations using media that do not support the growth of normal human mammary epithelial cells suggested that they represented the neoplastic component of the tumor specimen. To confirm this and to further characterize these cells, karyotype analysis was carried out. At passage five, SUM-102PT cells had a diploid karyotype with two clonal cytogenetic abnormalities: a reciprocal translocation involving chromosomes 5 and 12 and a second translocation involving chromosomes 6 and 16. The latter change was confirmed by fluorescence *in situ* hybridization using chromosome-specific probes (data not shown). Thus, the karyotype of these cells was consistent with their origin from an early-stage breast cancer that is only minimally invasive. Extended culture of SUM-102PT cells in the three different media resulted in further cytogenetic abnormalities, and these changes are summarized in Fig. 2. Despite the fact that these cells are cultured under slightly different conditions, all of the cells exhibited common marker chromosome abnormalities, indicating their origin from the same population of primary breast cancer cells.

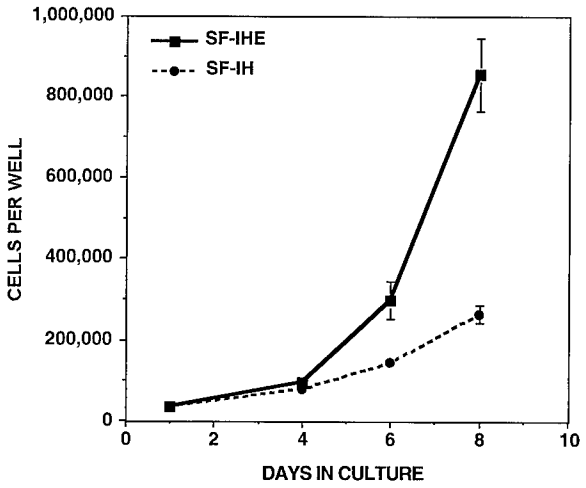


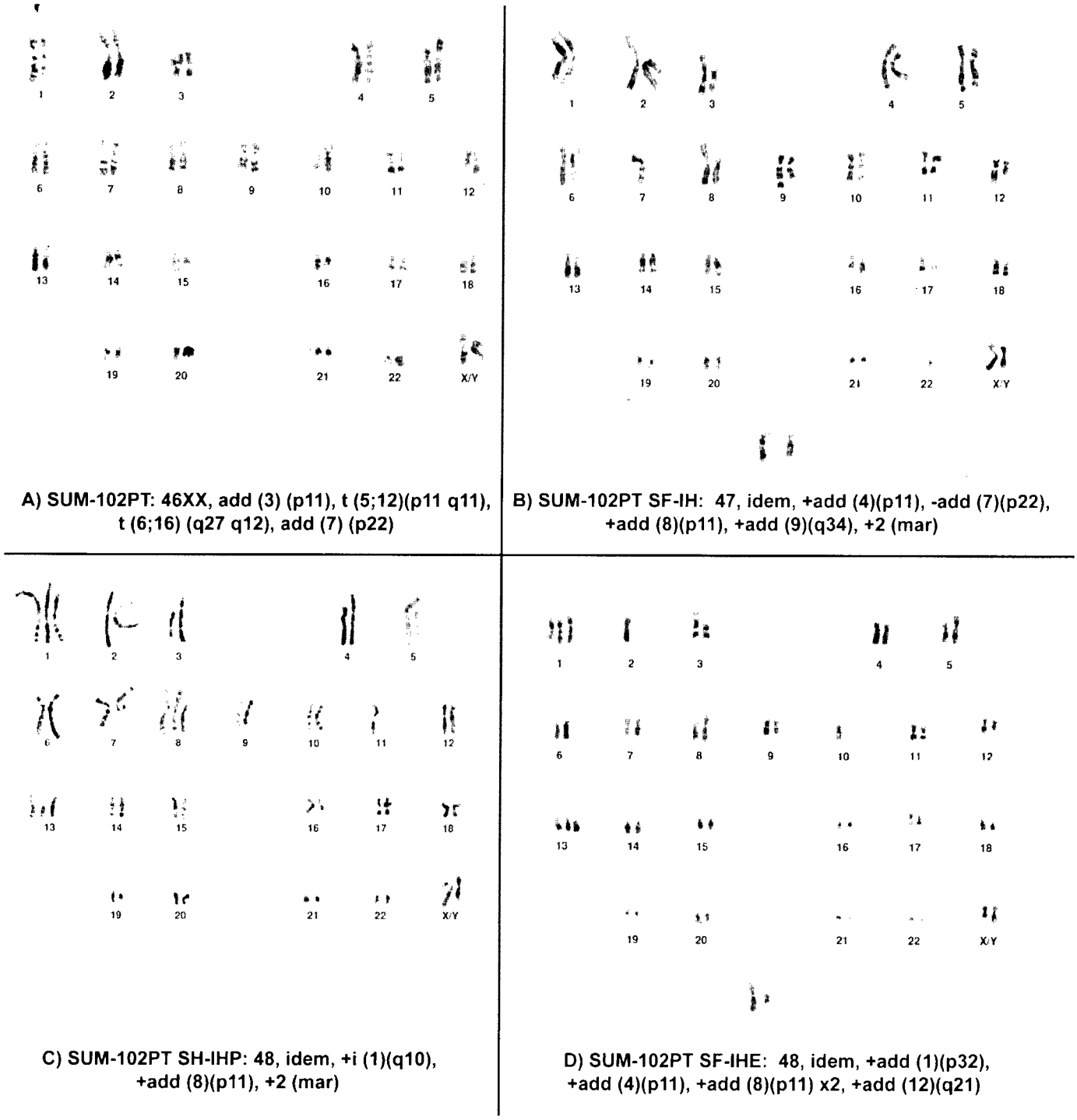
Fig. 1. Effect of Exogenous EGF on the proliferation of SUM-102PT cells normally grown without EGF. SUM-102PT cells cultured in SF-IH medium were seeded at 5×10^4 cells per 35-mm well and grown for 7 days in the presence or absence of 10 ng/ml EGF. *Points*, the mean number of cells per well for triplicate wells; bars, SD.

Southern blot analysis of SUM-102PT cells for alterations in genes commonly amplified in human breast cancer did not reveal amplifications of the *erb*B-2, c-*myc, Prad-1, FGFR-1, FGFR-2,* and *FGFR-4* genes (data not shown). These cells also do not overexpress nuclear p53 as examined by immunocytochemistry. However, exposure of these cells to 5 Gy of γ radiation resulted in the expression of nuclear p53 within 2 h of exposure, thus confirming the wild-type p53 status of these cells (data not shown).

Experiments were carried out to examine cytokeratin expression of the SUM-102PT cells. The vast majority of human breast cancers are of luminal cell origin (26, 27). Therefore, SUM-102PT cells were examined for expression of luminal cytokeratins by immunocytochemistry. SUM-102PT cells were negative for keratin-19 but were positive for the luminal cytokeratins keratin-8 and keratin-18 (Fig. 3). The neoplastic cells present in the biopsy specimen were also keratin-19 negative, whereas neighboring normal luminal cells stained positively for this keratin (data not shown). This pattern of luminal keratin expression indicates that the neoplasm arose from luminal cells. The expression of keratins 8 and 18 without expression of keratin 19 suggests that this tumor may have arisen in a duct rather than from a terminal duct lobular unit, because most luminal cells of the ducts are positive for keratins 8 and 18 but negative for keratin 19 (26, 27). This pattern of luminal cytokeratin expression may also be reflective of the apocrine metaplasia of the neoplastic cells of this specimen.

Role of EGFR Activation in SUM-102PT Cell Growth. To evaluate further the role of EGFR activation in the growth of the three SUM-102PT sublines, Western blots were prepared from membrane protein of these cells, and blots were probed with either an EGFR antibody or with the anti-phosphotyrosine antibody PY-20. Fig. 4 shows that all three sublines were positive for EGFR expression by Western blot. Interestingly, the highest levels of EGFR expression were detected in cells cultured in the SF-IHP medium. The lowest levels of expression, both at the message and protein levels, were in the subline cultured continuously in EGF-containing medium. Incubation of these cells in the absence of EGF for 24 h resulted in a dramatic increase in the level of cell surface EGFR (data not shown).

The phosphotyrosine Western blot also had a prominent M_r 170,000 band, suggesting that EGFR is activated in these cells. As in the EGFR Western blot, the most intense p-Tyr-M_r 170,000 band was detected in the SF-IHP cells. This result suggests that even the cells cultured continuously in the SF-IHP medium require EGFR activation for growth. To test this hypothesis further, all three sublines were cultured in their regular SF medium in the presence or absence of a neutralizing EGFR antibody. As expected, the EGFR antibody blocked growth of the cells cultured in the SF-IH and SF-IHE media. This antibody also blocked the proliferation of the cells cultured in the SF-IHP medium, consistent with the presence of constitutively tyrosine-phosphorylated EGFR in these cells (Fig. 5*A*). Similar experiments were then performed using an EGFR kinase inhibitor that has been shown to be a potent and specific inhibitor of the enzymatic activity of the EGFR (28). The EGFR kinase inhibitor, PD157655,



A) SUM-102PT: 46XX, add (3) (p11), t (5;12)(p11 q11), t (6;16) (q27 q12), add (7) (p22)

B) SUM-102PT SF-IH: 47, idem, +add (4)(p11), -add (7)(p22), +add (8)(p11), +add (9)(q34), +2 (mar)

C) SUM-102PT SH-IHP: 48, idem, +i (1)(q10), +add (8)(p11), +2 (mar)

D) SUM-102PT SF-IHE: 48, idem, +add (1)(p32), +add (4)(p11), +add (8)(p11) x2, +add (12)(q21)

Fig. 2. Karyotype analysis of SUM-102PT and derivative strains cultured in the three growth media. SUM-102PT cells cultured in SF-IHP media were karyotyped at passage five (*A*). These cells exhibited clonal abnormalities in chromosomes 3 and 7 and two reciprocal translocations involving chromosomes 5 and 12, and 6 and 16. Serial subculture of these cells through 50 passages resulted in additional cytogenetic abnormalities. The cells in all three media exhibited all of the abnormalities originally identified in early-passage cells, indicating their common origin, but had acquired additional alterations (*B–D*). The specific nature of the additional alterations was different for the cells cultured in the different media.

like the EGFR antibodies, potently inhibited the growth of SUM-102PT cultured in SF-IHP medium (Fig. 5*B*). By contrast, this inhibitor had no effect on growth of SUM-52PE human breast cancer cells, which do not express EGFR (15). Thus, we conclude that SUM-102PT cells, regardless of the media they are cultured in, require activation of the EGFR for growth *in vitro* under SF conditions.

Autocrine/Juxtacrine Activation of EGFR. The results presented above show that SUM-102PT cells require EGFR activation for growth. Therefore, experiments were performed to examine the *EGFR* gene copy number and expression levels in these cells. Southern blot analysis of genomic DNA from SUM-102PT cells indicated that the *EGFR* gene is not amplified in these cells (Fig. 6*A*). However, Northern blot analysis did show that EGFR is overexpressed at the message level in SUM-102PT cells cultured in SF-IHP, relative to MCF-10A normal human mammary epithelial cells (Fig. 6*B*). Interestingly, EGFR message levels were low, but detectable, in cells cultured continuously in the SF-IHE medium, suggesting that the

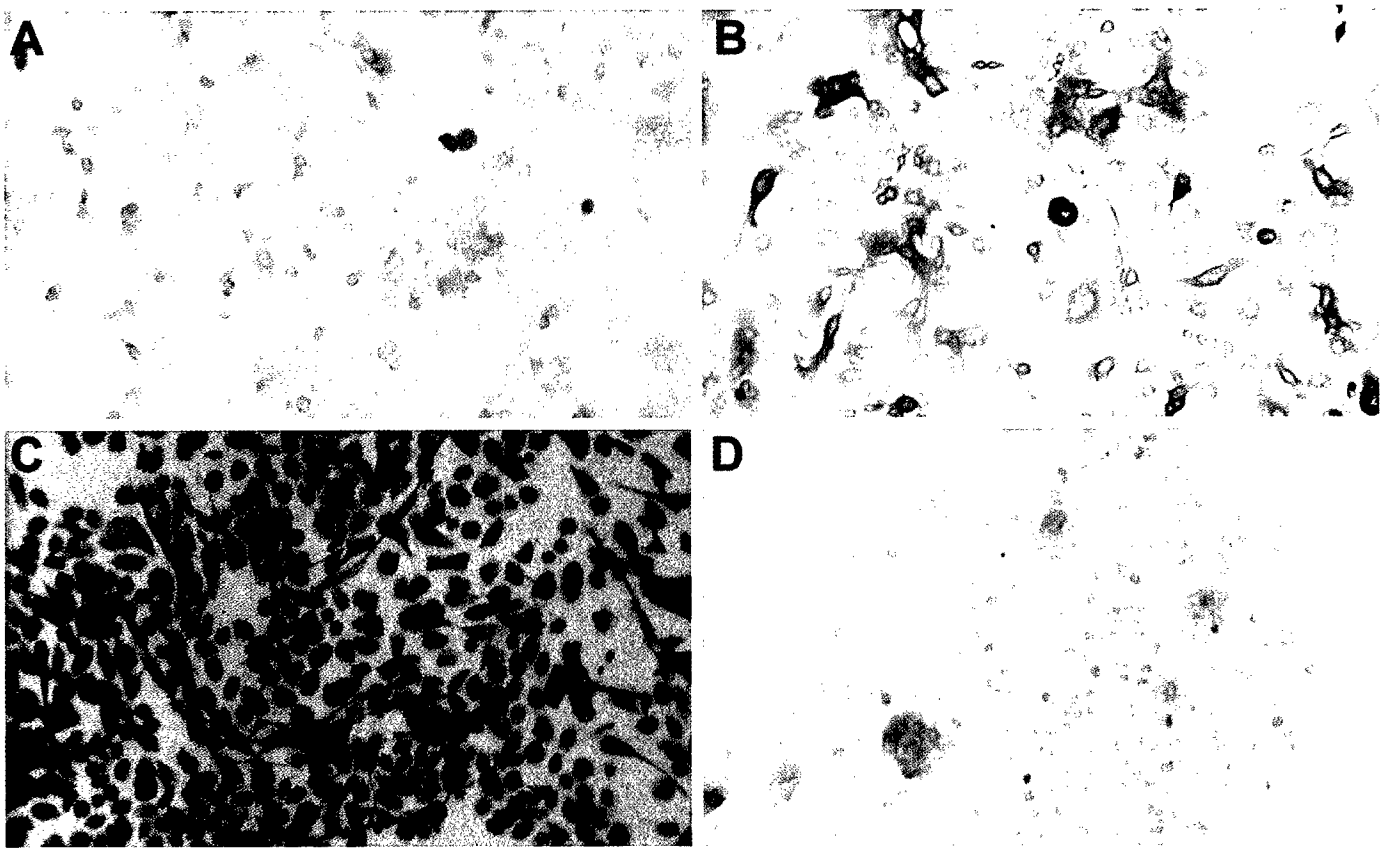


Fig. 3. Cytokeratin profile of SUM-102PT cells by immunocytochemistry. Photomicrographs of SUM-102PT cells immunostained using antibodies against luminal cytokeratins. *A*, keratin-8 immunostaining; *B*, keratin-18 immunostaining; *C*, pan-keratin immunostaining; *D*, keratin-19 immunostaining. Note positive staining of keratin-8 and keratin-18 but negative staining of keratin-19, indicating luminal origin despite keratin-19 negativity.

continuous presence of EGF in the culture medium influences the steady-state levels of EGFR message in these cells.

Given that the EGFR is not amplified in SUM-102PT cells, it is likely that the constitutive tyrosine phosphorylation of EGFR seen in the SF-IHP subline was due to an autocrine- or juxtacrine-acting growth factor synthesized by the cells. Therefore, experiments were carried out to determine if SUM-102PT cells were producing an EGF-like factor that was responsible for the EGFR activation. We have shown previously that MCF10A cells, which are completely dependent on EGF for proliferation, can be used as sensitive indicator cells for EGF-like activity in conditioned media (29, 30). The data in Fig. 7 show that conditioned medium obtained from SUM-102PT cells, grown continuously in SH-IHP medium, stimulated growth of MCF-10A cells under SF, EGF-free conditions.

Northern blot experiments were then carried out to screen for production of known EGF-like ligands by SUM-102PT cells. These experiments indicated that SUM-102PT cells produced low levels of transforming growth factor α message, as did the control MCF10A cells (data not shown). By contrast, neither SUM-102PT nor MCF10A cells expressed detectable levels of amphiregulin (data not shown). However, SUM-102PT cells cultured continuously in either the SF-IH or SF-IHP media expressed readily detectable levels of HB-EGF message, whereas MCF10A cells and SUM-102PT cells grown in SF-IHE medium did not (Fig. 8*A*). The finding of HB-EGF expression in the SUM-102PT cells that showed high levels of constitutive EGFR activation suggests that this factor acts as an important autocrine/juxtacrine factor for SUM-102PT cells.

To confirm that HB-EGF protein was expressed by these cells, immunocytochemical analysis was carried out using an HB-EGF antibody. The results of this analysis are shown in Fig. 8*B* and indicate

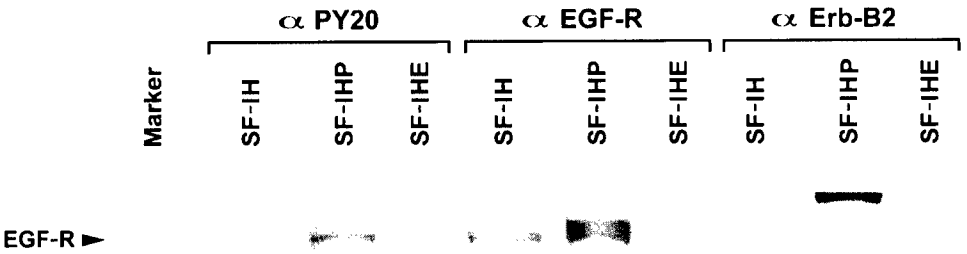


Fig. 4. EGFR and phosphotyrosine Western blot of SUM-102PT cells. One hundred μg of membrane protein from SUM-102PT cells that were grown in each of the three media were electrophoresed through a 7% SDS-polyacrylamide gel, transferred to Immobilon-P membranes, and probed with: anti-phosphotyrosine (α *PY20*); anti-EGFR (α *EGF-R*); or anti-Erb-B2 antibodies (α *Erb-B2*). Note the overexpression of the M_r 170,000 protein in the anti-EGFR blot, which co-migrates with the band in the anti-phosphotyrosine blot but is distinct from the M_r 185,000 Erb-B2 band.

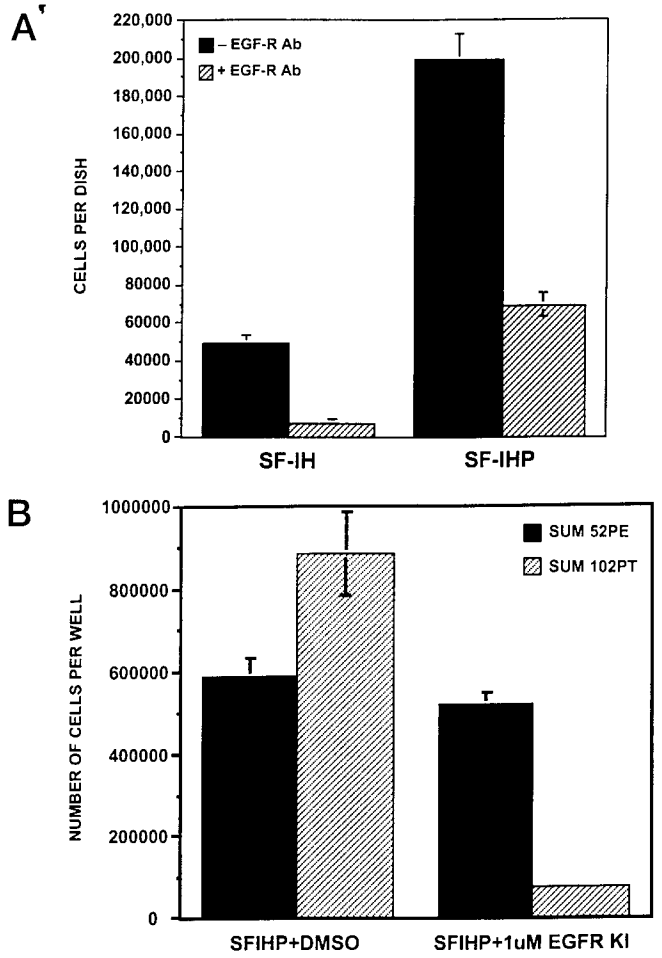


Fig. 5. The effect of a neutralizing EGFR antibody (*A*) or an EGFR kinase inhibitor (*B*) on the growth of SUM-102PT cells in EGF-free media. Cells were seeded at 3.5×10^4 cells per 35-mm well and cultured in SF-IH or SF-IHP medium. On days 1 and 4, one-half the wells in each group were also given 1 μg/ml of EGFR neutralizing antibody, Ab-1, or exposed to PD157655 at a 1 μM concentration. As a control for the kinase inhibitor experiment, EGFR-negative SUM-52PE cells were also tested for growth inhibition by this compound. Values are the mean number of cells/well for triplicate wells after 7 days in culture; *bars*, SD.

positive cell-surface expression of HB-EGF in these cells. To extend this observation, we took advantage of the fact that HB-EGF, in its membrane-bound form, is the DT receptor (31, 32). Cells that contain HB-EGF bind DT in the EGF-binding domain with subsequent internalization and cytotoxicity. Fig. 9 shows that SUM-102PT cells were sensitive to DT and were killed in a concentration-dependent manner. This result indicates that HB-EGF is present on the surface of these cells.

To confirm that HB-EGF was responsible for the mitogenic activity of the conditioned medium, SUM-102PT cells were grown to near confluence, and 48-h conditioned medium was collected. The conditioned medium was passed over a heparin-agarose affinity column, and the flow-through fraction was collected and tested for mitogenic activity. The column was then eluted in three fractions containing increasing salt concentrations, and the eluates were desalted and tested for EGF-like mitogenic activity. The data in Fig. 9*A* show that the medium that passed through the column was devoid of mitogenic activity and that the 1.0 M salt eluate did contain EGF-like activity. Thus, the conditioned medium factor does have high affinity heparin-binding activity. Finally, an HB-EGF antibody, as well as a nontoxic analogue of DT (CRM-197), was tested for their ability to inhibit the growth of SUM-102PT cells. The data in Fig. 9*C* show that both of these reagents could, at least partially, block the proliferation of SUM-102PT cells. Thus, SUM-102PT cells synthesize, express on their cell surface, and secrete HB-EGF, which plays a role in the constitutive tyrosine phosphorylation of EGFR in SUM-102PT cells and the growth of these cells in EGF-free medium.

STAT-3 Is Constitutively Activated in SUM-102PT Cells. The STAT family of transcription factors has been shown to be a component of the signal transduction pathway activated by EGFR in some cells (33–36). We, therefore, investigated whether this pathway was activated in the SUM-102PT cell line. Electrophoretic mobility shift assays were performed using the high affinity SIE of the c-fos promoter as a probe for activated STAT proteins (24, 37, 38). When SUM-102PT nuclear extracts were incubated with SIE oligonucleotides, several protein-oligonucleotide complexes formed, which resulted in distinct bands seen in *Lane 1* of Fig. 10. Competition with excess unlabeled SIE oligonucleotides, but not a scrambled oligonucleotide of the same base pair composition, abrogated complexes specific for the SIE, as seen in Fig. 10, *Lanes 2* and *3*. To determine

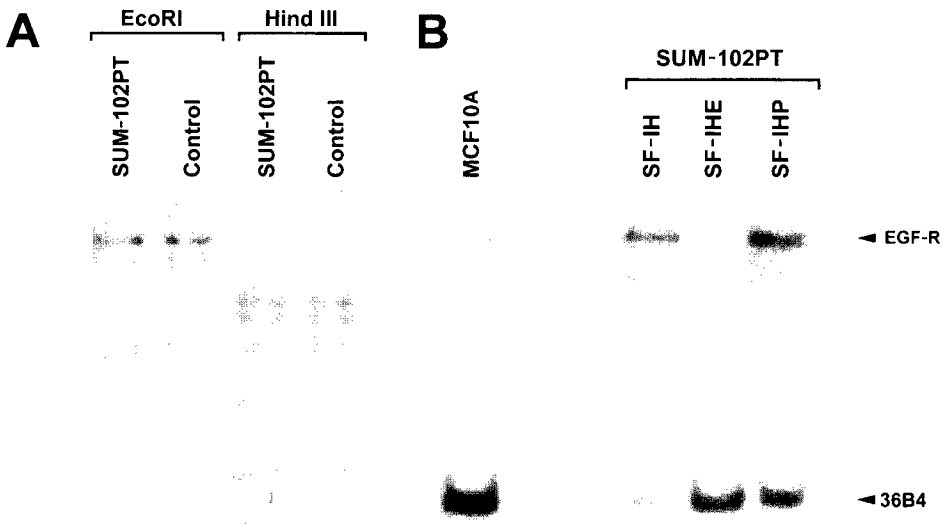


Fig. 6. *EGFR* gene copy number and expression levels in SUM-102PT cells. *A*, Southern blot of SUM-102PT Cells. DNA was isolated from SUM-102PT cells, digested with *Eco*RI or *Hin*dIII, separated electrophoretically through 0.8% agarose gels, transferred to nylon membranes, and probed with full-length EGFR cDNA. *Lane 1*, SUM-102PT digested with *Eco*RI. *Lane 2*, *Eco*RI-digested DNA obtained from cells known to contain no amplification of the *EGFR* gene (1315 MO2). *Lane 3*, SUM-102PT digested with *Hin*dIII. *Lane 4*, *Hin*dIII digest of the control DNA. *B*, EGFR Northern blot of SUM-102PT cells. Total RNA was isolated from SUM-102PT cells grown in the three media or MCF10A cells grown in SF media with insulin, hydrocortisone, and EGF. Twenty μg of RNA were electrophoresed through 1.5% agarose, transferred, and probed with the full-length EGFR cDNA. *Lane 1*, MCF-10A. *Lane 2*, SUM-102PT cultured in SF-IH medium. *Lane 3*, SUM-102PT cultured in SF-IHE medium. *Lane 4*, SUM-102PT cultured in SF-IHP medium. 36B4 was used to standardize relative amounts of RNA (*bottom panel*). Note the intensity of the EGFR band in the SUM-102PT cells grown in the absence of EGF relative to MCF-10A cells (which are known to have high levels of EGFR), despite overloading of the MCF-10A, as indicated by comparing the 36B4 signal.

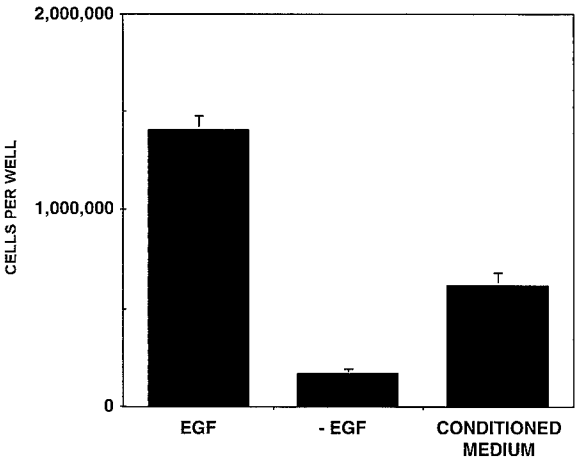


Fig. 7. Bioassay of EGF-like activity in SUM-102PT conditioned medium. MCF-10A cells, which are dependent on EGF for growth, were used as indicator cells to assay for secreted EGF-like ligands in conditioned medium from SUM-102PT cells. MCF-10A cells were seeded at 5×10^4 cells/well in 35-mm wells and grown with or without 10 ng/ml EGF or with 48 h conditioned media (50% v/v) from SUM-102PT cells grown in the absence of exogenous EGF. Media were changed every 2 days. Values are the mean number of cells per well for triplicate wells after 7 days; *bars*, SD.

the identity of proteins involved in the complexes, antibodies to specific STAT proteins were co-incubated with nuclear extracts and SIE oligonucleotide. Fig. 10, *Lane 4*, demonstrates supershift of the slowest migrating complex, indicating that this complex contains STAT-3. None of the bands were supershifted with STAT-1 antibodies.

Because SUM-102PT cells express high levels of constitutive EGFR activity and STAT-3 activity, a similar electrophoretic mobility shift assay was performed with nuclear extracts from MDA-468 breast cancer cells. MDA-468 cells have an amplification of the EGFR and express high levels of constitutively tyrosine-phosphorylated EGFR protein (39, 40). The data in Fig. 10 show that MDA-468 cells also express the activated STAT-3 homodimer.

We next looked at the SIE binding activity of MCF10A nuclear extracts to see whether STAT-3 complex formation was a common feature of EGFR signaling in human mammary epithelial cells. MCF10A cells, which have similar amounts of activated EGFR to the SUM-102PT cells grown in the presence of EGF, demonstrated different complex formation (Fig. 10). Specifically, MCF10A cells did not contain the STAT-3 complex seen in SUM-102PT cells. Thus, SUM-102PT cells express constitutively activated STAT-3, which was not present in MCF10A cells grown under identical conditions.

DISCUSSION

In this report, we describe the isolation and characterization of a new human breast cancer cell line from a primary breast neoplasm. There are a number of characteristics of this cell line that distinguish it from other breast cancer cell lines isolated previously by ourselves and others. This cell line was isolated from a primary tumor specimen following neo-adjuvant chemotherapy. The patient was originally diagnosed with locally advanced disease based on both mammographic and ultrasound findings and was, therefore, given chemotherapy prior to definitive surgery. Interestingly, the diagnosis of the final tumor specimen after mastectomy indicated that the tumor consisted mostly of carcinoma *in situ* with a microinvasive component. The original size of the primary neoplasm was the result of a large cystic component of the mass. Thus, this cell line was isolated from an early stage breast cancer, and the karyotype of SUM-102PT cells was consistent with the histological pattern of the neoplasm. SUM-102 cells were near diploid at early passages and exhibited only two discernible chromosome abnormalities: a reciprocal translocation involving chromosomes 6 and 16; and a second translocation involving chromosomes 5 and 12. At later passages, the karyotype became slightly more complex, but even these karyotypes were near diploid and much more normal in appearance than karyotypes from other primary tumor and metastatic cell lines that we have isolated (10, 41). One unusual feature of this breast cancer cell line is that it is negative for keratin-19 while being positive for the luminal cytokeratins keratin-8 and keratin-18. It is clear that human breast cancer arises, almost exclusively, from luminal epithelial cells and most often from the terminal duct lobular unit. Most, but not all, of these luminal cells express keratin-19. However, keratin-19-negative luminal cells do express two other luminal cytokeratins, *i.e.*, keratins-8 and 18, and the SUM-102PT cells are positive for these. Thus, although the keratin-19 negativity of these cells is unusual, it is not without precedent in human breast cancer (26, 27).

SUM-102PT cells were first isolated using two selective media that do not support growth of normal human mammary epithelial cells. One of these media was supplemented with insulin and hydrocortisone, while the other medium also contained progesterone. The SF-IHP medium yielded cells with a more compact morphology that grew more rapidly than cells isolated in the SF-IH medium. The fact that the

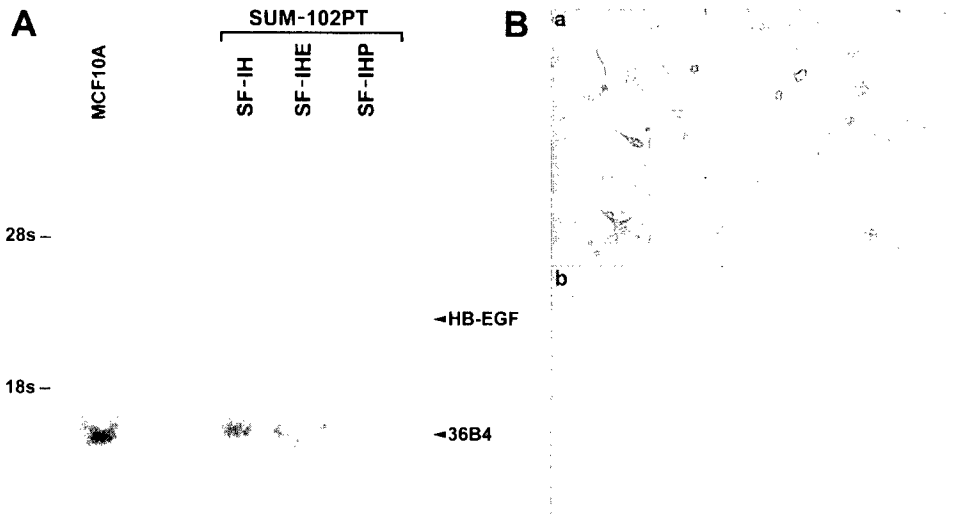


Fig. 8. HB-EGF expression in SUM-102PT cells. *A*, HB-EGF Northern Blot of SUM-102PT. Total RNA was isolated from SUM-102PT cells grown in the three media or MCF10A cells grown in SF media with insulin, hydrocortisone, and EGF. Twenty μg of RNA were electrophoresed through 1.5% agarose, transferred, and probed with a 1.1-kb HB-EGF probe. *Lane 1*, MCF-10A. *Lane 2*, SUM-102PT cultured in SF-IH medium. *Lane 3*, SUM-102PT cultured in SF-IHE medium. *Lane 4*, SUM-102PT cultured in SF-IHP medium. 36B4 was used to standardize relative amounts of RNA (*lower band*). Note that HB-EGF message is expressed in the SUM-102PT cells, which showed high levels of activated EGFR. *B*, HB-EGF immunocytochemistry of SUM-102PT Cells. Photomicrographs show SUM-102PT cells immunostained using antibody to HB-EGF (*A*) or nonimmune IgG control (*B*).

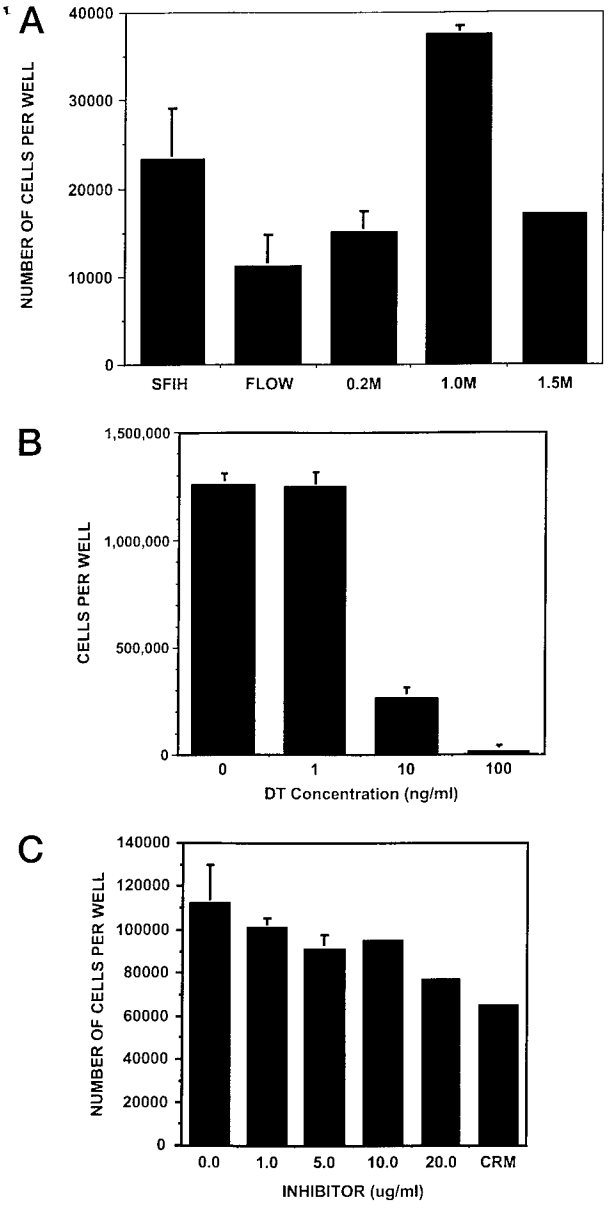


Fig. 9. The role of HB-EGF in autocrine/paracrine growth of SUM-102PT cells. *A*, heparin-agarose chromatography of SUM-102PT conditioned medium. Conditioned medium that either flowed through the column or was eluted with varying salt concentrations was tested for EGF-like mitogenic activity on MCF-10A cells as described in Fig. 7. *B*, effect of DT on the growth of SUM-102PT cells cultured in SF-IHP medium. *C*, effect of HB-EGF antibodies or a nontoxic analogue of DT (CRM-197) on the growth of SUM-102PT cells cultured in SF-IHP medium. For the experiments shown in *B* and *C*, SUM-102PT cells were seeded at 5×10^4 cells in six-well plates with DT or antibody at the concentrations shown. The CRM-197 was used at a concentration of 10 μg/ml. Media were changed every 2 days, and nuclei were isolated and counted after three media changes. Values are the average number of cells in triplicate wells of triplicate experiments: *bars*, SD.

cells grew continuously in EGF-free medium suggested that they were EGF independent for growth. However, the finding that cells grown in SF-IHP medium had constitutively tyrosine-phosphorylated EGFR suggested that EGFR activation was required for the growth of these cells. Consistent with this hypothesis was the observation that the cells cultured in the SF-IH medium were highly responsive to exogenous EGF, which stimulated their growth to a rate comparable to the SF-IHP cells. The observation that both EGFR antibodies and EGFR kinase inhibitors completely blocked the growth of SUM-102PT cells, regardless of what medium they were cultured in, confirmed the dependency on EGFR activation for growth of these cells.

SUM-102PT cells express high levels of EGFR, both at the message and protein levels, without amplification of the *EGFR* gene. Thus, these cells appear to be representative of a large subset of human breast cancer cells that overexpress EGFR in the absence of gene amplification. Overexpression of EGFR without gene amplification occurs in approximately 30% of cases and has been observed repeatedly to be associated with poor outcome (16–23). This is potentially of mechanistic significance because overexpression of a growth factor receptor may be a central factor in transforming an autocrine or paracrine loop from a physiological to a pathological process.

HB-EGF appears to be the ligand that drives constitutive tyrosine phosphorylation of EGFR and proliferation of SUM-102PT cells cultured continuously in SF-IHP medium. SUM-102PT cells express HB-EGF mRNA and protein detectable at the cell surface. EGF-like mitogenic activity was also observed in conditioned medium obtained from these cells, and this activity was retained on a heparin-agarose affinity column. The 1.0 M salt eluate from this column contained the EGF-like mitogenic activity. DT effectively killed SUM-102PT cells, indicating the presence of HG-EGF on the cell surface. However, antibodies to HB-EGF, or a nontoxic analogue of DT, were only marginally effective at blocking the growth of these cells. The relatively poor ability of these reagents to block growth of these cells may be a reflection of true cellular autonomy of HB-EGF or may reflect the inability of this particular antibody to block the interaction of HB-EGF with the EGFR, especially when the growth factor itself is located predominantly on the cell surface. In this regard, its worth noting that these same antibodies were only poorly effective at blocking the growth of epidermal keratinocytes, which have also been shown to synthesize and secrete HB-EGF (42).

Heparin-binding epidermal like growth factor was originally isolated from macrophage-like U937 cells as a M_r 22,000 heparin-binding factor that is mitogenic for smooth muscle cells (43). HB-EGF was found to have 40–50% homology to other known EGF-like

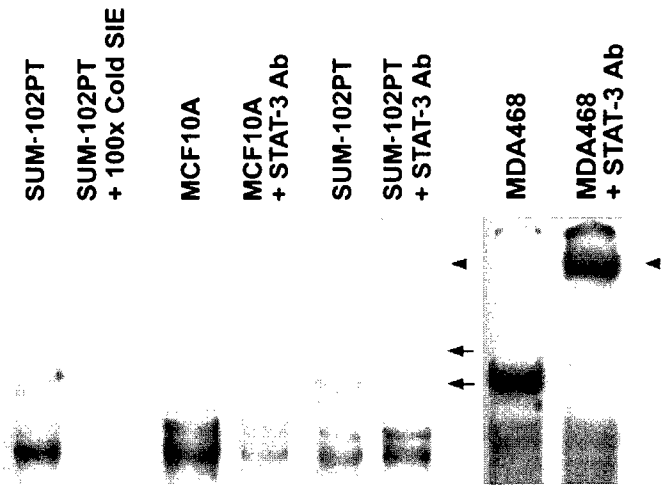


Fig. 10. Electrophoretic mobility shift assay of SIE binding proteins in nuclear extracts from SUM-102PT, MDA-468, and MCF10A cells. Nuclear extracts were incubated with end-labeled SIE oligonucleotides (5′-AGCTTCATTTCCCGTAAATCCCTA) in the presence or absence of cold competitor oligonucleotide or STAT antibody and run on 7.5% polyacrylamide gels. *Lane 1*, SUM-102PT nuclear extract. *Lane 2*, SUM-102PT nuclear extract with 100-fold unlabeled SIE competitor. *Lane 3*, MCF-10A nuclear extract. *Lane 4*, MCF-10A nuclear extract with STAT-3 antibody. *Lane 5*, SUM-102PT nuclear extract. *Lane 6*, SUM-102PT nuclear extract with STAT-3 antibody. *Lane 7*, MDA-468 nuclear extract. *Lane 8*, MDA-468 nuclear extract with STAT-3 antibody. Note that the slowest migrating complex in the SUM-102PT and MDA-468 extracts are supershifted with antibody to STAT-3 (*arrowheads*), whereas the MCF10A cells contain a slower migrating complex that is distinct from the SUM-102PT complex and does not supershift with antibody to STAT-3 (*arrows*).

factors, having the conserved six-cysteine domain characteristic of EGF family members, and is a ligand for the EGFR (44). HB-EGF was subsequently discovered to function as a membrane-bound precursor as well as a secreted molecule (45, 46). In its membrane-bound form, proHB-EGF functions as the DT receptor. DT binds proHB-EGF in the EGF-like region, which allows internalization of DT, with resultant toxicity, and inhibits binding of HB-EGF to EGFR (31, 32, 47, 48). HB-EGF expression has been shown in many tissues, particularly lung, skeletal muscle, brain, and heart (45). In keratinocytes, HB-EGF has been implicated in autocrine/paracrine interactions; normal human keratinocytes have EGFRs, express HB-EGF mRNA, and respond to exogenous HB-EGF by proliferation (42). HB-EGF has also been demonstrated in mammary carcinoma cell lines (46), but its functional role in these lines is unknown. To our knowledge, the results reported here represent the first report of HB-EGF autocrine/paracrine activity in a human breast carcinoma cell line.

It is interesting that the HB-EGF autocrine/paracrine loop appeared to function most efficiently in cells grown in progesterone-containing medium. These cells had a faster doubling time and higher levels of EGFR activation than cells isolated and grown without progesterone. Progesterone has been reported to increase HB-EGF mRNA levels in rat uterine stromal cells (49), and progesterone with estradiol increased HB-EGF mRNA in mouse uterus (50). The precise role that progesterone plays in regulating the growth of SUM-102PT cells remains to be elucidated.

We also found that SUM-102PT cells express a constitutively active STAT-3 complex that is not detectable in MCF-10A human mammary epithelial cells cultured under the same conditions. The STAT family of proteins was first identified by their involvement in the IFN response (38, 51, 52), which leads to STAT complex formation, tyrosine phosphorylation, and translocation to the nucleus, where the complex binds to enhancer elements of genes activated by the IFNs (53, 54). Tyrosine kinase-activated receptors, such as the EGFR, have also been found to use the STAT pathway. EGFR-activated STAT complexes typically consist of homo- and heterodimers of the STAT-1 and STAT-3 proteins. These activated STAT complexes ultimately bind the SIE of the c-fos promoter, thus contributing to immediate-early gene expression (55, 56). We found that SUM-102PT cells contain constitutively activated STAT-3 homodimeric complexes that bind the c-fos SIE with high affinity. In previous reports, STAT-3 complex formation has been demonstrated in EGF-stimulated hepatocytes (35) in cells with very high levels of EGFR, such as A431 cells, (52, 57), or in EGFR transfectants (58). Although it is possible that the level of EGFR activation in SUM-102PT cells is itself sufficient for STAT-3 complex formation, an alternative explanation is that STAT-3 is being activated by other kinases. Experiments underway are aimed at determining the tyrosine kinase directly responsible for STAT-3 activation in SUM-102PT cells and other EGFR-positive HBC cells.

The significance of STAT activation in human breast cancer is unknown. Previous studies demonstrated that STAT-3 is constitutively activated by the src oncoprotein, providing the first link between a specific oncoprotein and activation of STAT signaling pathways (24). STAT-DNA binding activity has been demonstrated in breast cancer nuclear extracts but not in normal breast tissue histology samples (59). Our finding that SUM-102PT cells grown in EGF had constitutive STAT activation, whereas MCF10A cells that were grown under the same conditions did not express activated STAT-3, suggests that STAT activation is part of an abnormal signal transduction process in some malignant breast cells. Consistent with this hypothesis was our observation that MDA-468 human breast cancer cells, which have an amplification of the *EGFR* gene and which also exhibit constitutive EGFR activity, also express high levels of the activated STAT-3 complex.

In our laboratory, we have recently developed 12 new human breast cancer cell lines. These lines have been examined for STAT-3 activation, and the results indicate that STAT-3 activation is associated with EGFR expression in these human breast cancer cells.[4] Thus, constitutive STAT-3 activation appears to be a property of breast cancer cells with constitutive EGFR activation.

In summary, the SUM-102PT human breast cancer cell line represents a novel cell line that can be cultured under defined conditions *in vitro*. These cells may be representative of that subset of human breast cancer characterized by estrogen receptor negativity and overexpression of EGFR in the absence of gene amplification. The data presented here indicate that the overexpression of the EGFR is of direct importance to the altered growth potential of these cells.

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Role of pp125^{fak} in morphogenesis of human mammary epithelial cells and in anchorage-independent growth of human breast cancer cells

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Running title: pp125^{fak} activation in human breast cancer cells

Abstract

Our laboratory has developed twelve human breast cancer cell lines from primary and metastatic sites. Here, we demonstrate that these breast cancer cell lines exhibit constitutively tyrosine phosphorylated and enzymatically active pp125^{fak}. In monolayer, the activation status of pp125^{fak} in breast cancer cells is dramatically elevated over that exhibited by normal mammary epithelial cells. Tyrosine phosphorylation of pp125^{fak} was induced to high levels by culturing the normal mammary epithelial cells in *Matrigel*, which induces the cells to undergo morphogenesis. Thus tyrosine phosphorylation of pp125^{fak} is a regulated process in normal mammary epithelial cells, but is constitutive in breast cancer cells. High levels of pp125^{fak} activity in breast cancer cells were associated with high levels of pp60$^{c\text{-}src}$ activity. Exposure of the breast cancer cell lines to an active inhibitor of *src* kinase significantly reduced the level of tyrosine phosphorylated proteins in these cells but had no effect on the level of pp125^{fak} tyrosine phosphorylation. When cultured in soft agar, normal human mammary epithelial cells rapidly and uniformly lost viability; whereas, the breast cancer cell lines survived in suspension, and some of the breast cancer cell lines grew to form large colonies. Thus constitutive activation of pp125^{fak} results in preferential survival of human breast cancer cells under anchorage-independent conditions but is not sufficient to stimulate colony formation in soft agar.

Introduction

Attachment of mammary epithelial cells to their extracellular matrix is essential for normal tissue homeostasis. Signals sent from the extracellular matrix receptors on the cell surface take part in the regulation of cell growth, differentiation, morphogenesis, and survival (Ashkenas *et al.*, 1996). Attachment of human mammary epithelial (HME) cells occurs, in part, through cell surface receptors known as integrins, that are composed of heterodimers of one alpha subunit and one beta subunit (Hynes, 1992). The specific combination of alpha and beta subunits indicates the type of substrate to which the cells will attach (Ashkenas *et al.*, 1996).

Recently, Howlett, *et al.* (1995) showed that normal HME cells, when placed in a laminin-rich basement membrane matrix, develop acinar structures similar to mammary ducts; whereas, human breast cancer (HBC) cells form amorphous colonies when grown in the same matrix. Abrogation of HME morphogenesis occurred when the cells were grown in the presence of anti-β_1-integrin antibodies; however, the anti-integrin antibodies had no effect on the growth of the tumor cells. These investigators also demonstrated that HME cells in the presence of anti-integrin antibodies undergo apoptosis, but the tumor cells do not. This observation was later duplicated by Meredith, *et al.* (1993) with HME cells and by Frisch and Francis (1994) with HT1080 cells and MDCK cells. All of these data suggest that normal human epithelial cells require signals derived from attachment to extracellular matrix to survive in three-dimensional culture, whereas tumor cells do not.

In 1992, Schaller, *et al.* identified a putative substrate of activated pp60$^{c\text{-}src}$, a protein of about 125 kD, as a membrane-associated tyrosine kinase that is phosphorylated on tyrosine, serine, and threonine. The kinase was termed focal adhesion kinase, or FAK. In fibroblasts, the *fak* protein is localized to focal adhesions (Hildebrand *et al.*, 1993; Schaller *et al.*, 1992) by binding to the β_1-integrin (Hildebrand *et al.*, 1993). About the same time as Schaller, *et al.* identified pp125^{fak}, it was demonstrated that signaling through the fibronectin/β1 integrin receptor system caused an increase in tyrosine phosphorylation of an approximately 120 kD protein (Guan *et al.*, 1991). It was later shown by Guan and Shalloway (1992) that the 120 kD protein was identical to pp125^{fak}.

Because pp125^{fak} associates with a known proto-oncogene product, pp60$^{c\text{-}src}$ (Schaller *et al.*, 1992; Xing *et al.*, 1994) and is involved in signaling through integrin-regulated pathways, pp125^{fak} may play a role in facilitating tumor development. Weiner, *et al.* (1993) examined the expression of *fak* in breast cancer cells. They found that 9 of 11 primary breast cancers contained elevated levels of *fak* mRNA as compared to benign breast tissue, and all 4 metastatic tumors studied had high levels of *fak* expression. Later, Owens, *et al.* (1995) demonstrated that 88% of invasive and metastatic breast tumors studied had significantly elevated pp125^{fak} levels compared to normal tissue from the same patient. These data suggest that *fak* overexpression plays a role in breast cancer development and invasive potential.

Since blocking β1-integrin-mediated signaling triggers apoptosis, the activation state of pp125^{fak} may play a role in the regulation of programmed cell death. These data suggest that dysregulated pp125^{fak} activation may be partly responsible for certain transformed properties of HBC cells and may protect against apoptosis.

Our laboratory has recently established twelve new HBC cell lines from various stages of breast cancer. These cell lines were used to investigate the role of pp125^{fak} activation in the ability of these cells to survive and grow under anchorage-independent conditions. The data presented here suggest that constitutive pp125^{fak} activation plays a role in the ability of breast cancer cells to survive under conditions that induce apoptosis in normal breast epithelial cells.

Results

Human breast cancer cell lines

This laboratory has recently established twelve new human breast cancer cell lines from primary and metastatic human breast tumors. Descriptions of three of the cell lines used in this study, namely SUM-44PE, SUM-52PE, and SUM-102PT, have been published previously (Ethier *et al.*, 1993; Ethier *et al.*, 1996; Sartor *et al.*, 1997). Experiments were also carried out with a more recently isolated HBC cell line, SUM-149PT. SUM-149PT was established from a primary inflammatory, infiltrating ductal carcinoma of the breast. The cells from this tumor are estrogen receptor negative (ER^-) and highly epidermal growth factor receptor positive ($EGFR^+$); however, they do not require EGF for growth *in vitro*. These breast cancer cells do not have amplifications of any of the following oncogenes: erbB-2, c-*myc*, FGFR-1, -2, and -4, or Prad-1. However, these cells do overexpress nuclear p53 protein, and SSCP analysis confirmed the presence of a mutation in exon 7 (data not shown) . Table 1 summarizes characteristics of the HBC cells used in these studies.

HBC cell lines have increased pp125^{fak} activity

Weiner, *et al.* and Owens, *et al.* (Weiner *et al.*, 1993; Owens *et al.*, 1995) showed increased *fak* mRNA and pp125^{fak} protein in human breast tumors compared to normal tissue. Based on these observations, we postulated that the HBC cell lines would have elevated levels of *fak* protein compared to normal human mammary epithelial cells. Western blot analysis was carried out to compare *fak* protein levels and activation status in our HBC cell lines to normal HME cells. The data in Figure 1A show that all of the HBC cell lines had approximately the

same amount of pp125^{fak} as the normal human mammary epithelial cell line MCF-10A. These data did not rule out an increase in pp125^{fak} activation in HBC cells relative to normal cells, so protein blots of immunoprecipitates of pp125^{fak} were probed with anti-phosphotyrosine antibodies. Figures 1B and 1C show that all of the HBC cell lines examined had increased tyrosine phosphorylation of pp125^{fak} compared to normal mammary cell controls. This suggests that HBC cells have higher levels of pp125^{fak} activity than normal mammary cells. To confirm that pp125^{fak} was activated to higher levels in HBC cells compared to normal cells, pp125^{fak} immunoprecitates were subjected to *in vitro* kinase assays. The results shown in Figure 1D confirm the increased enzymatic activity of the *fak* protein in HBC cells compared to pp125^{fak} from MCF-10A controls.

HME cells in three-dimensional culture have increased pp125^{fak} activity

To determine if pp125^{fak} present in normal HME cells could be activated by stimulation with extracellular matrix, pp125^{fak} activation was studied under conditions that simulate normal mammary epithelial cell growth and morphogenesis; HME cells were grown within a basement membrane matrix, *Matrigel*. Under these conditions, HME cells grew into three-dimensional structures exhibiting ductal branching and formation of acini (Figure 2B). Anti-phosphotyrosine-probed protein blots of pp125^{fak} immunoprecipitates from HME cells revealed a dramatic increase in levels of tyrosine-phosphorylated pp125^{fak} in cells grown in *Matrigel* (Figure 3). Thus, in HME cells, pp125^{fak} is tyrosine phosphorylated as a result of interaction with the basement membrane proteins in a manner that does not occur in monolayer. By contrast, the HBC cell lines all exhibited high levels of constitutive pp125^{fak} tyrosine phosphorylation when grown in monolayer (Figure 1C).

HBC cells have increased pp60$^{c\text{-}src}$ activity

Since pp125^{fak} had higher activity in the HBC cells than in the normal controls grown in monolayer, and pp125^{fak} has been shown to be activated in cells expressing the highly active pp60$^{v\text{-}src}$, we investigated whether pp60$^{c\text{-}src}$ was activated in HBC cells compared to controls. The levels of expression, tyrosine phosphorylation, and activation of pp60$^{c\text{-}src}$ were examined. The HBC cell lines and the control cells all had approximately the same amount of pp60$^{c\text{-}src}$, except SUM-44PE cells which expressed lower levels (Figure 4B). In addition, most of the normal and HBC cells contained tyrosine phosphorylated pp60$^{c\text{-}src}$ (Figure 4C) However, tyrosine phosphorylation on pp60$^{c\text{-}src}$ can either activate or inactivate the enzymatic activity of the protein (Kmiecik & Shalloway, 1987; Cooper & MacCauley, 1988). Therefore, the activity of pp60$^{c\text{-}src}$ was investigated using an *in vitro* kinase assay. Data shown in Figure 4D indicate that all of the HBC cells had increased pp60$^{c\text{-}src}$ activity relative to the control MCF-10A cells. Thus, for all of the HBC cells studied, there was a positive association between the activation of pp125^{fak} and pp60$^{c\text{-}src}$.

Increased pp60$^{c\text{-}src}$ activity is not responsible for increased pp125^{fak} activity in HBC cells

Since activated pp125^{fak} associates with activated pp60$^{c\text{-}src}$ (Schaller *et al.*, 1994), it is not known if one kinase directly activates the other. To investigate the relationship between activation of pp125^{fak} and pp60$^{c\text{-}src}$, experiments were performed using a recently developed kinase inhibitor that is active against *src* kinase with minimal activity against pp125^{fak} (PD164199). The *in vitro* IC 50 values

for this inhibitor are 24 nM for pp60$^{c\text{-}src}$ and 50 μM for pp125^{fak}. Control cells and HBC cell lines were cultured with the inhibitor for 14 and 24 hr. Whole cell lysates were prepared after incubation with the inhibitor, and the amount of phosphotyrosine-containing proteins was determined by anti-phosphotyrosine western blot (Figure 5A). In the presence of the inhibitor, the levels of phosphotyrosine-containing proteins decreased dramatically in SUM-44PE, SUM-52PE, and SUM-149PT cells. In the control MCF-10A and in the SUM-102PT cells, the *src* kinase inhibitor was less effective. Although the overall amount of phosphotyrosine-containing proteins decreased in HBC cells in response to the *src* kinase inhibitor, the level of tyrosine-phosphorylated pp125^{fak} was unaffected after 14 hr incubation with the inhibitor (Figure 5B). These data are consistent with a model in which the activation of pp125^{fak} is upstream of pp60$^{c\text{-}src}$ activation.

HBC cells containing activated pp125^{fak} survive under anchorage-independent conditions

A hallmark of cellular transformation is the ability of cells to produce colonies in an anchorage-independent manner (Freedman & Shin, 1974). In order to assess the ability of the HBC cells to grow under anchorage-independent conditions, the HBC cell lines, HME cells, and MCF-10A cells were grown for three weeks in soft agar suspension. HME and MCF-10A cells do not form colonies in soft agar (Figure 6). By contrast, some of the HBC cell lines formed large colonies detectable at the standard diameter of 2 mm (Freedman & Shin, 1974) (Figure 7, middle panel). The HBC cells that did not form large colonies (Figure 6) did survive in soft agar and formed small colonies of only a few cells each (Figure 7, right panel). This is in stark contrast to HME cells which quickly and uniformly lost viability within one week in soft agar. After three weeks in soft agar, no visible HME or

MCF-10A cells were observable (Figure 7, left panel). Thus, HBC cells with activated pp125^{fak} were able to survive under anchorage-independent conditions; whereas, those without pp125^{fak} activation rapidly lost viability. These results suggest that pp125^{fak} activation is necessary for survival in the absence of matrix attachment but is not sufficient to stimulate rapid growth of cells under anchorage-independent conditions.

Discussion

The interaction of the extracellular matrix (ECM) with epithelial cells in influencing cell growth and morphogenesis has been studied in both rodent and mammalian cells for some time. ECM is deposited by the epithelial cells and stromal fibroblasts (Ashkenas *et al.*, 1996). Activation of specific signaling pathways via interactions of epithelial cell surface molecules with ECM are required for three dimensional growth of normal human and rodent mammary epithelial cells (Ashkenas *et al.*, 1996). HME cells grown in three-dimensional culture in the basement membrane *Matrigel* form structures reminiscent of fully differentiated aveoli *in vivo*. This is in contrast to HME cells grown on tissue culture plates which have the characteristic epithelial cell cobblestone appearance. Howlett, *et al.* (1995) demonstrated the importance of a particular signaling pathway for human mammary epithelial cells grown in three-dimensional culture. Blockage of the cell surface receptor β1-integrin's interactions with the extracellular matrix using an anti-β1-integrin antibody caused the cells to undergo apoptosis. In contrast, HBC cells grown under the same conditions did not organize into three-dimensional structures in this basement membrane matrix and did not undergo apoptosis when exposed to the anti-β1-integrin antibody.

It is known that β1-integrin binding to ECM activates a pathway that results in activation of pp125*fak* (Guan & Shalloway, 1992). pp125*fak* is a cytoplasmic tyrosine kinase (Schaller *et al.*, 1992) that binds β1-integrin in the cytosol through a sequence in its carboxy terminus (Hildebrand *et al.*, 1993). It was previously shown in primary uncultured human breast cancers that *fak* mRNA and protein levels are elevated relative to surrounding normal mammary epithelia (Owens *et al.*, 1995; Weiner *et al.*, 1993). This suggests that constitutive activation of pp125*fak* may be an

important aspect of HBC growth, particularly since these cells are not in contact with basement membrane (Albrechtsen *et al.*, 1986). The data obtained in our experiments support this hypothesis. We found that normal HME cells require interactions with components of *Matrigel* in order to activate pp125^{fak} (Figure 3). By contrast, all of the HBC cell lines examined had constitutively activated pp125^{fak} when grown in monolayer (Figure 1). Three more recently isolated HBC cell lines (SUM-159PT, SUM-185PE, SUM-190PT) were also found to have high levels of tyrosine phosphorylated phosphorylated pp125^{fak} (data not shown). Thus 7 of 7 HBC cell lines examined express high levels of constitutively active pp125^{fak}.

The protein tyrosine kinase pp60$^{c\text{-}src}$ is known to interact with pp125^{fak} in its active state (Calalb *et al.*, 1995). This interaction takes place when pp125^{fak} is activated and autophosphorylated, creating a high affinity binding site for activated pp60$^{c\text{-}src}$ (Schaller *et al.*, 1992; Schaller *et al.*, 1994; Xing *et al.*, 1994; Calalb *et al.*, 1995). Because both proteins require activation for this interaction to occur, it is not known whether pp125^{fak} activates pp60$^{c\text{-}src}$ or vice versa. In our HBC cell lines, we have demonstrated higher levels of activated pp125^{fak} and pp60$^{c\text{-}src}$ compared to normal cell controls (Figures 1 and 4). However, an inhibitor active against *src* kinase had no effect on pp125^{fak} tyrosine phosphorylation (Figure 5B), suggesting that pp60$^{c\text{-}src}$ does not activate pp125^{fak} in these cells.

The HBC cell lines developed in our laboratory can be classified into several categories based on molecular alterations (i.e. EGFR+/ER-, erb-B2 overexpressing, p53 mutant, etc.). However, until now, no characteristic has been observed uniformly in all of the HBC cell lines. The data presented here indicate that pp125^{fak} activation and pp60$^{c\text{-}src}$ activation, and their correlation with anchorage-

independent survival, represent common characteristics of our entire panel of HBC cell lines.

There is suggestive evidence in the literature that pp125*fak* activation is required for anchorage independent growth of transformed cells (Ruoslahti & Reed, 1994). Our data indicate that constitutive pp125*fak* activation is important for survival in the absence of matrix-derived signals but is not sufficient for formation of large colonies in soft agar. This interpretation is based on the observation that HME cells failed to survive in soft agar; whereas, the HBC cell lines, with activated pp125*fak*, survived under these conditions (Figure 7). In addition, only a subset of the HBC cell lines formed large colonies in soft agar (Figures 6 and 7). Our results are consistent with those from other laboratories that have demonstrated a role for pp125*fak* activation in blocking apoptosis in the absence of integrin signaling (Frisch *et al.*, 1996; Hungerford *et al.*, 1996).

By definition, malignant epithelial cells are those that can invade through the basement membrane and continue to grow and disseminate in the absence of signals from the extracellular matrix. Our results suggest that constitutive activation of pp125*fak* is part of an altered signaling pathway that is involved in the matrix-independent growth potential of human breast cancer cells.

Materials and Methods

Cell culture: The base medium for MCF-10A, SUM-44PE, and SUM-102PT cells was Ham's F12 media supplemented with 0.1% bovine serum albumin, 0.5 μg/ml fungizone, 5 μg/ml gentamycin, 5 mM ethanolamine, 10 mM HEPES, 5 μg/ml transferrin, 10 μM 3,3',5-triiodo-L-thyronine (T_3), 50 μM selenium, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. The base medium for HME, SUM-52PE and SUM-149PT was Ham's F12 supplemented with 5% fetal bovine serum (FBS), 0.5 μg/ml fungizone, 5 μg/ml gentamycin, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. MCF-10A and SUM-102PT cell medium was further supplemented with 10 ng/ml EGF. HME cell medium was further supplemented with 10 ng/ml EGF, and 100 μg/ml cholera toxin. All cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

HME cells were grown on collagen-coated tissue culture plates or in *Matrigel* (Collaborative Biomedical Products, Bedford, MA). Cells grown in *Matrigel* were plated in a 50/50 (v/v) mixture of *Matrigel* and HME medium. Cells were grown for two weeks prior to use for further experiments.

Immunoprecipitations and protein blots: Cells were lysed in a buffer consisting of 20 mM Tris·HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, and 50 μg/ml leupeptin. Protein concentrations were equalize using the Løwry method (Bradford, 1976). Equal amounts of protein were either loaded on 10% SDS-PAGE or immunoprecipitated with the appropriate antibody (pp125^{fak}: Catalog #F15020, Transduction Labs, Lexington, KY; pp60$^{c\text{-}src}$: Catalog #OPO7, Calbiochem, Cambridge, MA). Antibody was incubated with lysate for 1 hr at 4°C. The immune complex was bound to protein A/G beads (Calbiochem, Cambridge, MA) for 40 min at 4°C.

Immunoprecipitates were washed 2 times with phosphate buffered saline (PBS) containing 1% Triton X-100, 2 times with PBS containing 0.5% Triton X-100, and 2 times with PBS. Læmmeli sample buffer (Sambrook *et al.*, 1989) was added and the samples were boiled. Equal amounts were loaded onto 7.5% SDS-PAGE (for α-pp125*fak* immunoprecipitates) or 10% SDS-PAGE (for α-pp60$^{c\text{-}src}$ immunoprecipitates). Separated proteins were blotted to PVDF membrane and probed with either α-pp125*fak*, α-pp60$^{c\text{-}src}$, or α-Ptyr$_{4G10}$ (Catalog #05-321, Upstate Biotechnology, Inc., Lake placid, NY).

Kinase assays: α-pp125*fak* or α-pp60$^{c\text{-}src}$ immunoprecipitates were washed twice in lysis buffer and once in the appropriate kinase assay buffer (pp125*fak*: 50 mM Tris·HCL, pH 7.4, 10 mM $MnCl_2$; pp60$^{c\text{-}src}$: 100 mM PIPES, pH 6.8, 20 mM $MnCl_2$). Immunoprecipitates were mixed with prewarmed kinase assay buffer containing 20 μM ATP, 10 μCi γ-^{32}P-ATP, and the appropriate artificial substrate (pp125*fak*: 0.5 μg/μl poly(Glu:Tyr)$_{4:1}$ (Sigma Chemical Co., St. Louis, MO); pp60$^{c\text{-}src}$: 5μg/μl acid denatured enolase (Cooper *et al.*, 1984)). The reactions were allowed to proceed for 20 min at 30°C, stopped with the addition of Læmmeli sample buffer, and boiled. Reactions were then separated on 10% SDS-PAGE, blotted to PVDF, and probed for pp125*fak* or pp60$^{c\text{-}src}$ to be sure each sample had equivalent amounts of the kinase. Blots were then exposed to film at -80°C for 2 hrs to 2 days.

SRC kinase inhibitor assays: MCF-10A and HBC cells were incubated with or without 5 μm *src* kinase inhibitor (PD164199; Parke-Davis Pharmaceutical Research, a division of Warner-Lambert Co.) for 14 or 24 hours. Cells were lysed in lysis buffer. For whole cell lysates, protein concentrations of the lysates were equalized for all of the samples. For pp125*fak* Immunoprecipitations, protein concentrations were equalized within a cell type before pp125*fak* was immunoprecipitated as above.

Proteins were separated on 7.5% SDS-PAGE, blotted to PVDF, and probed with α-$Ptyr_{4G10}$

Soft agar assays: Six-well dishes were coated with a mix of the appropriate medium for the cell line being studied and 0.5% Bactoagar. Cells were plated at $1x10^3$, $1x10^4$, $1x10^5$ cells/well in a mixture of appropriate medium and 0.3% Bactoagar. Cells were fed every other day for 3 wks, stained with 500 μg/ml ρ-iodonitrotetrazolium violet (Catalog #I-8377, Sigma Chemical Co., St. Louis, MO) overnight, counted, and photographed.

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Table 1

Molecular Characteristics of the HBC Cell Lines

Cell Line	Oncogene Amp.*	EGFR	erbB-2	erbB-3	erbB-4	p53 (IHC)
SUM-44PE	**FGFR-1**	–	+	+	+	+ (c)
SUM-52PE	**FGFR-1 & 2**	–	+	+	+	+ (c)
SUM-102PT	**none**	+++	+	+	–	–
SUM-149PT	**none**	+++	+	+	–	+ (n)

*Oncogenes examined by Southern blot: erbB-2, c-myc, Prad-1, FGFR-1, 2, 4, EGFR
c=cytoplasmic staining, n=nuclear staining

Figure Legends

Figure 1: HBC cell lines contain increased pp125^{fak} activity. Proteins from whole cell lysates were separated on SDS-PAGE, blotted to PVDF, and probed with α-pp125^{fak} antibody (A). pp125^{fak} was immunoprecipitated from control and HBC cells. Immunoprecipitated proteins were separated on SDS-PAGE, blotted to PVDF, and probed with α-pp125^{fak} antibody (B), α-Ptyr$_{4G10}$ (C), or used to phosphorylate (Glu:Tyr)$_{4:1}$ in *in vitro* kinase assays (D). The SUM-44PE cell line was not available for A and D.

Figure 2: HME cells exhibit differentiated morphology when grown in basement membrane. HME cells were grown either on collagen-coated tissue culture plates (A) or in the laminin-rich basement membrane *Matrigel* (B).

Figure 3: HME cells grown in *Matrigel* contain higher levels of pp125^{fak} activity than HME cells grown in two-dimensional culture. pp125^{fak} was immunoprecipitated from cell lysates of HME cells grown on tissue culture plates or in *Matrigel*, separated on SDS-PAGE, blotted to PVDF, and probed with α-Ptyr$_{4G10}$ antibody. Since the amount of pp125^{fak} tyrosine phosphorylation correlates with its activity, the pp125^{fak} from HME cells grown in *Matrigel* was more active than pp125^{fak} from HME cells in two-dimensional culture.

Figure 4: HBC cells have increased pp60$^{c\text{-}src}$ activity. Proteins from whole cells lysates or anti-pp60$^{c\text{-}src}$ immunoprecipitates were separated on SDS-PAGE, blotted to PVDF and probed with α-pp60$^{c\text{-}src}$ antibody (A and B), α-Ptyr$_{4G10}$ (C), or used to phosphorylate acid-denatured enolase in *in vitro* kinase assays (D).

Figure 5: pp125^{fak} activation is not dependent on pp60$^{c\text{-}src}$ activation in HBC cells. Proteins from whole cell lysates (A) or anti-pp125^{fak} immunoprecipitations (B) from cells incubated with or without *src* kinase inhibitor PD164199 were separated on SDS-PAGE, blotted to PVDF, and probed with α-Ptyr$_{4G10}$ antibody.

Figure 6: Anchorage-independent growth assay of HBC cell lines and normal HME cells. Control cell lines and HBC cell lines were grown in 0.3% agar for 3 weeks, stained, and colonies greater than 2 mm were counted.

Figure 7: The HBC cells containing increased pp125^{fak} activity survived under anchorage-independent conditions. HBC cells not capable of forming colonies did survive in soft agar in contrast to control cells which underwent apoptosis in soft agar. Examples of each cell type, apoptotic (MCF-10A), colony-forming (SUM-52PE), and surviving (SUM-44PE), are shown.

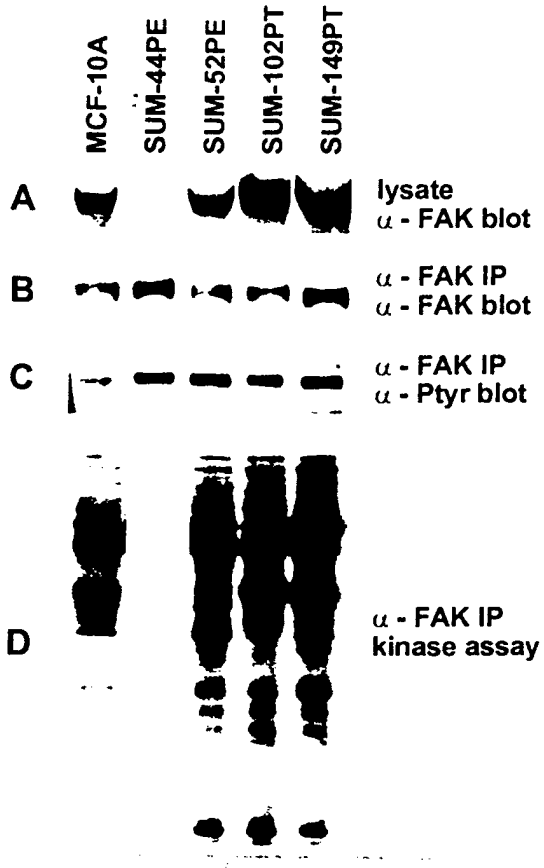

Figure 1

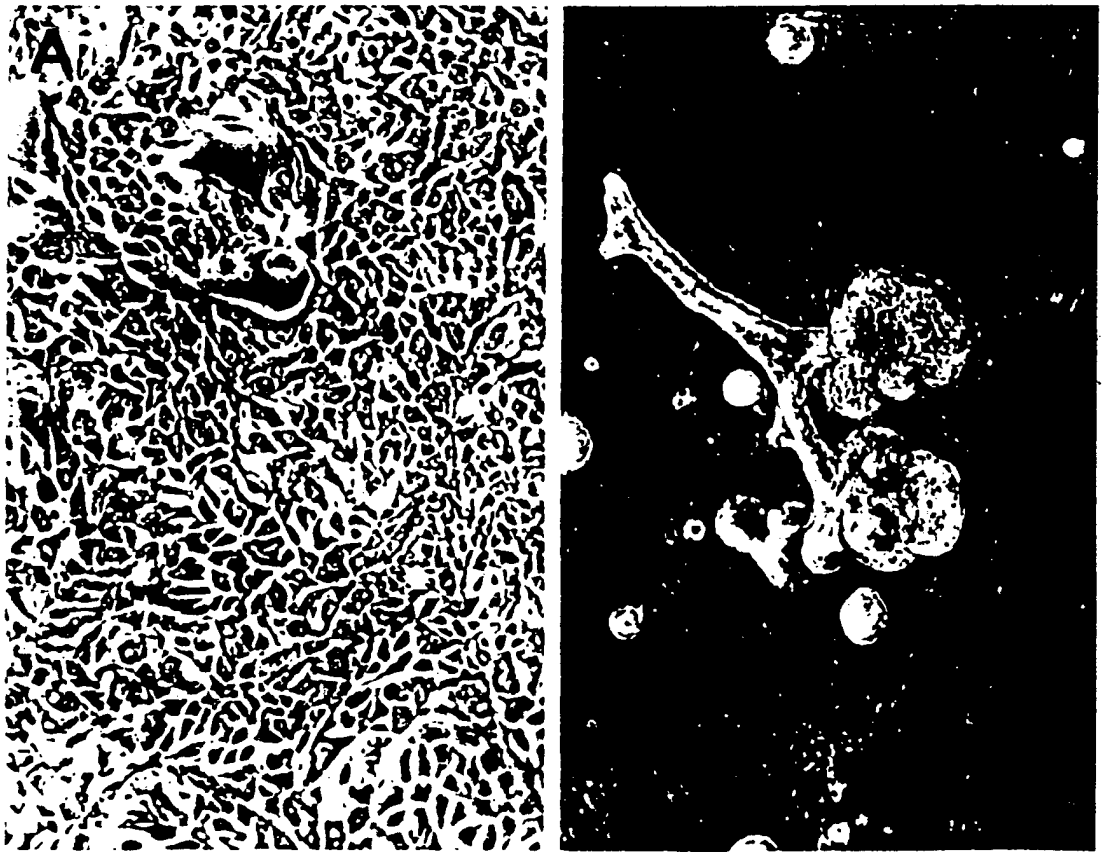

Figure 2

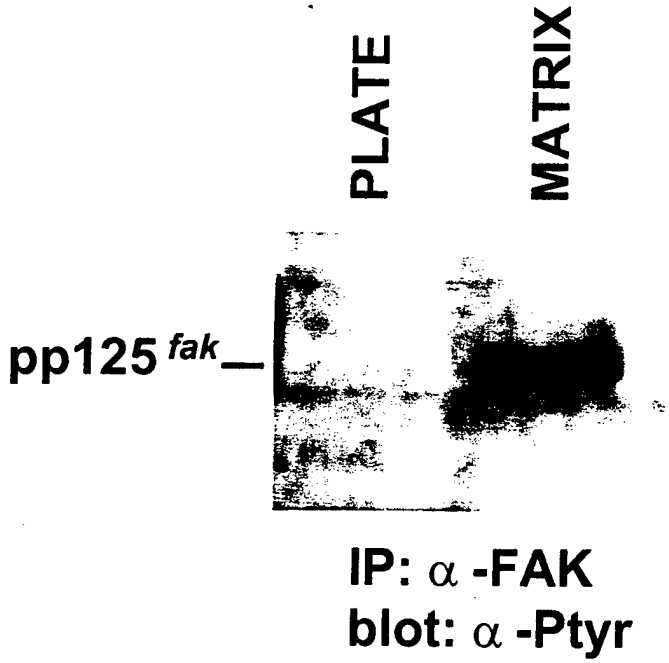

Figure 3

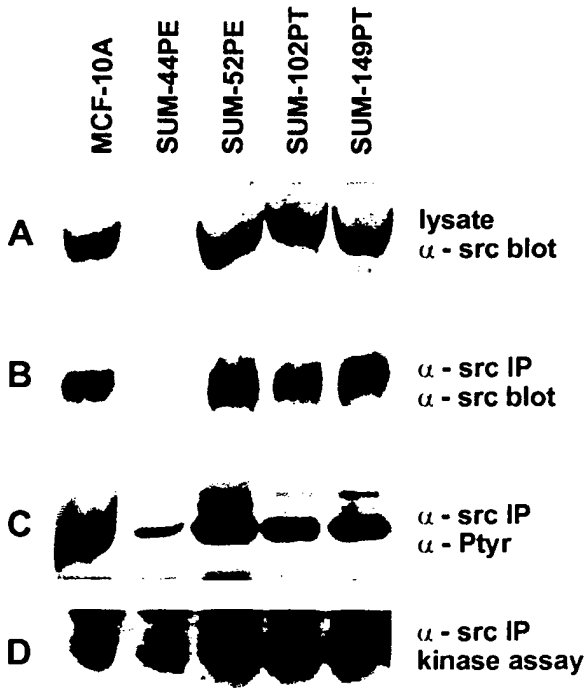

Figure 4

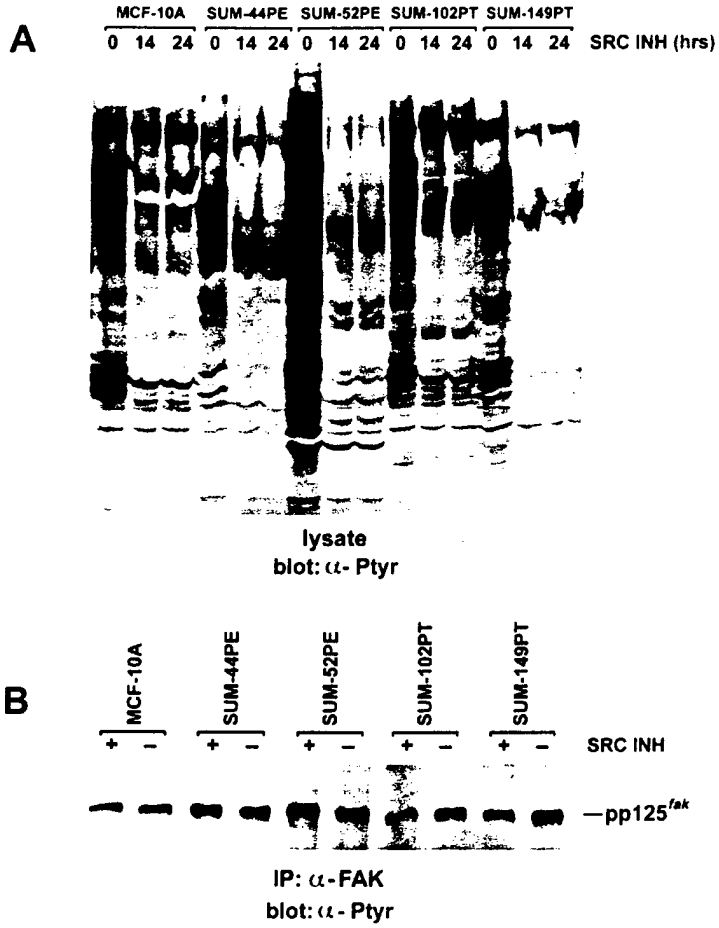

Figure 5

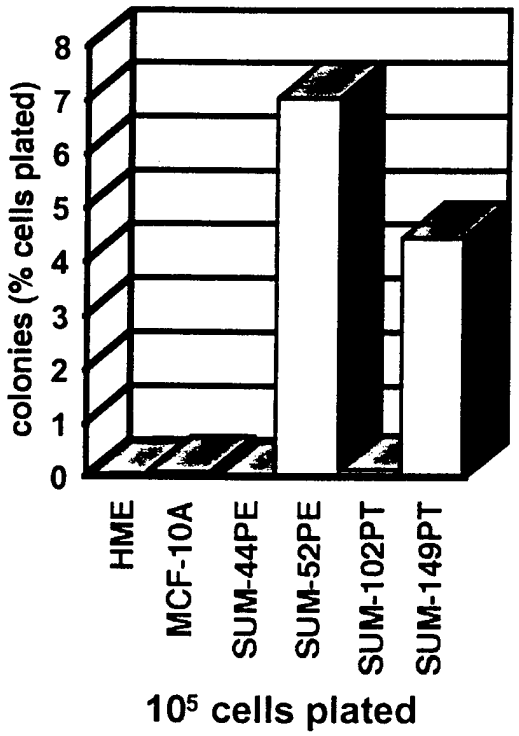

Figure 6

Figure 7